ANALYSIS OF MLH1 AND MSH2 IMMUNOHISTOCHEMISTRY IN COLORECTAL CARCINOMAS

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PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH

PEELAMEDU, COIMBATORE – 641004.

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CERTIFICATE

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This is to certify that the dissertation work entitled "ANALYSIS OF MLH1 AND MSH2 IMMUNOHISTOCHEMISTRY IN COLORECTAL CARCINOMAS" submitted by Dr. P.Pavethira is the work done by her during the period of study in the department of Pathology, PSG IMS & R from April 2010 to March 2013.This work was done under the guidance of Dr. Suma.B.Pillai, Associate Professor, Department of Pathology.

Dr. Suma.B.Pillai M. D
Associate Professor,
Department of Pathology,
PSG IMS & R.

Dr. Alamelu Jayaraman. M. D.
Professor & HOD,
Department of Pathology,
PSG IMS & R.

Dr. S.Ramalingam. M. D.
Principal,
PSG IMS & R,
Coimbatore – 641004.

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CONTENTS

INTRODUCTION

Globally the medical fraternity is facing a major challenge in the form of increasing incidence of colorectal carcinoma. Colorectal cancer is the 3rd most common malignancy reported worldwide. Countries like North America, Australia, and Europe are facing an increased incidence of colorectal carcinoma. [1] When compared to the western world, colonic cancer is less common in India. [2] Colorectal carcinomas are mostly associated with diet, genetics and environmental factors. With westernization of life style, India is facing an increase in incidence of colorectal cancer. [1]

Two different pathogenetic pathways are implicated in colorectal carcinomas. The microsatellite stable pathway (MSS) where there is inactivation of tumor suppressor genes like APC, p53, and DCC. The other pathway which plays a major role in colorectal carcinomas is inactivation of mismatch repair genes such as MLH1, MSH2, MSH6, PMS2, and MSH3. These belong to microsatellite instability pathway (MSI).

Literature states that virtually all the cases of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) / Lynch syndrome and 15% of sporadic cases of colorectal carcinomas have MSI. Mutation in two MMR genes, MLH1 and MSH2 accounts for majority of cases of HNPCC [1]

It is postulated that testing for MSI would serve two purposes:

1. It is a powerful tool to screen for HNPCC and therefore members of the family with HNPCC can benefit from clinical survey by colonoscopy.

2. Though MSI colorectal carcinomas are relatively insensitive to treatment with 5-flurouracil based chemotherapy, they have a better prognosis.[1]

Hence the knowledge of the MSI status in colorectal carcinoma cases would help the clinician to assess the prognosis and also to guide in therapy.

MMR deficient sporadic and hereditary colorectal carcinomas are frequently located in the ascending colon. These are large tumors and are usually of high grade. They also have peritumoral and intratumoral lymphocytic infiltrate which are implicated for better prognosis.

Though molecular testing is gold standard for MSI testing to diagnose mismatch repair gene defects there are several studies which suggest a >95% specificity for immunohistochemical analysis.

Molecular testing for MSI is laborious, time consuming, expensive and can be done only in selected diagnostic laboratories, whereas immunohistochemical method costs 14-28% less, less time consuming and can be done in any pathology laboratory.

Therefore we propose to study the deficiency of MMR protein expression by immunohistochemical method in a series of colorectal carcinoma cases reported from our institute and to correlate with various clinicopathological characteristics.

AIMS AND OBJECTIVES

1] To determine the frequency of loss of mismatch repair protein (MLH1 and MSH2) expression by immunohistochemical method in colorectal carcinomas.

2] To study its correlation with various clinical and histopathological characteristics and to determine if there is any significant association between the two.

REVIEW OF LITERATURE

Carcinoma of the colon is the third most common cancer and it accounts for 8.5% of cancers worldwide in 2000. About 945,000 new cases of colorectal carcinomas are being diagnosed every year. As per the statistical data by US Surveillance Epidemiology and End Results (SEER), the incidence of colonic carcinoma is 33.7 per 100,000 and that of rectal carcinoma is 12.8 per 100000. Both are common among males. [1] Incidence rate of colorectal carcinoma in Asia varies widely. It is uniformly low in all South Asian countries and high in developed Asian countries like Japan, South Korea and Singapore. [2]

In India the incidence rate is low and it represents about 2% of all the malignancies. Rectal carcinoma is more common when compared to colon cancer in India. Rural population based studies show very low incidence rate of colon carcinomas and high incidence rate of rectal carcinomas probably due to limited data collection.[3] Some population based studies show that there is a rising trend in the incidence of colorectal carcinoma in India.[4]

Colonic carcinomas occur most commonly in elderly men and the risk of acquiring colorectal carcinoma increases with age after 40 years of age. Family history also plays an important role in the etiology. Colorectal cancers may be sporadic or

familial. Sporadic cases have a multifactorial etiology such as dietary habits-western type diet, sedentary life style, long standing inflammatory bowel disease, pelvic irradiation. [1]

High intake of red meat can increase the risk of colorectal cancers, probably due to increased production of heterocyclic amines, stimulation of higher levels of fecal bile acids, increased production of oxygen free radicals and increased insulin levels. On the other hand intake of high vegetable and fiber diet prevents colorectal cancer. [1] Incidence of colorectal carcinoma among Indian immigrants in United States and United Kingdom is found to be high, suggesting a causal relationship with life styles and dietary habits. [4]

At least two distinct genetic pathways have been described in the development of familial colorectal cancers.

1. APC/β- catenin pathway associated with classic adenoma – carcinoma sequence.
2. Microsatellite instability (MSI) pathway with defects in DNA mismatch repair genes (MLH1, MSH2, MSH3, PMS2 and MSH6).

Mutation of APC gene causes Familial adenomatous polyposis. MSI is present in virtually all the cases of hereditary nonpolyposis colorectal cancer (HNPCC) and approximately 15% of sporadic cases. Mutation in two MMR genes, MLH1 and MSH2 accounts for majority of cases of HNPCC [5]

The presence of colorectal cancer in a first degree relative increases a person's lifetime risk for developing colorectal cancer from 1.8 –fold to as high as eightfold that of general population. The incidence of colonic neoplasm among first degree relatives of colorectal cancer patients ranges from 15% to 20%.The risk will be increased in those having more than one affected relative. This kind of familial clustering of colorectal cancer could be due to shared gene pool, a shared environment or a combination of both. [6]

Apart from dietary and genetic factors, other factors associated with colorectal carcinomas include diverticulosis, inflammatory bowel disease, socioeconomic factors, hormonal factors, radiation, gall stones & cholecystectomy. [7]

It was Burkitt who suggested the coexistence of diverticulosis and colorectal cancers and is more frequently observed among western population compared to Asian. Patients with inflammatory bowel disease exhibit an increased risk of developing colorectal carcinoma.[8] The incidence of colorectal carcinoma is 4 to 20 times greater in patients with inflammatory bowel diseases when compared to normal population. They also develop cancer outside of the GI tract. Several studies have shown an increased incidence of right sided colonic cancer in women of all ages and a relation with parity, which may protect against the development of colorectal cancer. It has also been found that 70% of colonic cancers are estrogen

receptor positive. [9] Women radiated for gynaecological malignancies have a relative high risk for subsequent colonic cancer of 2.0 to 3.6 times. [10]

The WHO sub classifies colorectal carcinoma as follows:

- Adenocarcinoma
- Mucinous adenicarcinoma
- Signet ring cell adenocarcinoma
- Small cell carcinoma
- Squamous cell carcinoma
- Adenosquamous carcinoma
- Medullary carcinoma
- Undifferentiated carcinoma

Most colorectal carcinomas are located in sigmoid colon and rectum. Recent trends show an increased incidence of proximal carcinomas. Tumors with high levels of microsatellite instability are more frequently located in cecum, ascending colon and transverse colon. [11]

The gross appearance of colorectal carcinomas may be polypoid, fungating (exophytic), ulcerating, stenosing or diffusely infiltrating. Generally in caecum they are bulky, exophytic and polypoidal, rarely causing obstruction, whereas the proximal colorectal carcinomas are most often infiltrative and ulcerating producing annular constricting tumors.

CONVENTIONAL ADENOCARCINOMA:

The diagnostic feature of colonic adenocarcinoma is the invasion through muscularis mucosae into sub mucosa. If the tumors have high grade morphological changes but fail to invade muscularis mucosa, the term intramucosal neoplasia is used. [11]

Most carcinomas have atypical cells arranged in glandular pattern with morphology ranging from well to moderately differentiated. Histological grading is mainly based on the architecture.

- Well differentiated adenocarcinoma (grade 1) exhibit glandular structures in >95% of tumor
- Moderately differentiated adenocarcinoma (grade2) has 50-95%

  glandular structures
- Poorly differentiated (grade 3) adenocarcinomas have 5-50% glandular

structures. In poorly differentiated tumors, cells are predominantly arranged in solid sheets and the cells exhibit loss of nuclear polarity and considerable nuclear pleomorphism.

- Undifferentiated grade (grade 4) has <5% glandular structures. When one portion of the tumor appears well differentiated while other area is poorly differentiated, the histological grade is assigned according to the least differentiated area found.[12]

MUCINOUS ADENOCARCINOMA:

When more than 50% of the tumor is mucinous, it is classified as mucinous adenocarcinoma. About 10 to 15% of colorectal carcinomas and 33% of rectal carcinomas are mucinous tumors. Histologically it is characterized by pools of extracellular mucin in which tumor cells are floating as acini or as single cells. This type has an association with high frequency microsatellite instability and often affects young patients. Mucinous carcinomas have a poor prognosis probably due to delay in diagnosis. These tumors show more extensive lymphnode involvement and are likely to invade adjacent viscera when compared to non mucinous adenocarcinomas. [12]

SIGNET RING CELL CARCINOMA:

This is a variant of mucinous adenocarcinoma characterized by the presence of signet ring cells (cells with abundant intracellular mucin pushing the nucleus to the periphery) in > 50% of the tumor. They represent about 0.5 to 1.0% of all colorectal carcinomas and usually occur in younger age group. Like mucinous adenocarcinoma, signet ring carcinoma also present at an advanced stage and has a poor outcome. [13] Some MSI tumors are of this type.

SQUAMOUS CELL CARCINOMA

Primary squamous cell carcinomas of colon are very rare. There are different theories about its histogenesis, many favour an origin from pluripotent stem cell. Other theory suggests its origin from metaplastic foci associated with chronic inflammation. [14] Microscopically it resembles squamous cell carcinoma of any other organ. Diagnosis of squamous cell carcinoma can be made only after excluding secondary metastasis from other sites.

ADENOSQUAMOUS CARCINOMA:

These are extremely rare neoplasm accounts for 0.06% of all colorectal cancers. Histologically it show features of both squamous and adenocarcinoma. Both these components can be seen either as separate components or admixed [1]

MEDULLARY CARCINOMA:

Medullary carcinoma is a recent entity recognized by WHO. Microscopically, it is composed of sheets of large polygonal cells having vesicular nuclei, prominent nucleoli and abundant eosinophilic cytoplasm. It is often associated with a dense lymphocytic infiltrate. It was first described by Jessurrun and coworkers as an undifferentiated carcinoma. These tumors are more likely to harbor mutations in KRAS and TP53 and defects in DNA mismatch repair gene. These tumors are common in women and occur in proximal colon. Medullary carcinomas have a more favorable prognosis. [15]

UNDIFFERENTIATED CARCINOMA:

They have variable morphology and lacks differentiation beyond their epithelial origin. These tumors are usually seen in association with MSI and have a poor prognosis. [11]

OTHER VARIANTS

CARCINOSARCOMA

Malignant tumors containing both carcinomatous and heterologous mesenchymal elements are termed as carcinosarcoma.

Other rare variants include choriocarcinoma, giant cell carcinoma, clear cell carcinoma, stem cell carcinoma and paneth cell- rich carcinoma [11]

Staging of colorectal carcinomas

Dukes' staging:[12]

This staging was proposed by dukes in 1937. By this staging the colorectal carcinomas are staged by the level of infiltration of the wall by the tumor as;

Stage A: Tumor confined to the bowel wall

Stage B: Tumor infiltrating the serosa or the perimuscular fat in areas without peritoneal covering.

Stage 3: Tumors with lymph node metastases

Astler and coller staging: [12]

This staging was proposed in the year 1957.

Stage A: Tumor confined to mucosa.

Stage B1: Tumor involving the muscularis propria without penetrating it.

Stage B2: Tumor penetrating muscularis propria

Stage C1: Tumor limited to bowel wall with lymph node metastases

Stage C2: Tumor penetrating the bowel wall and featuring lymph node metastases.

Now the currently used staging was the one proposed by World Health Organisation , taking into consideration of three factors such as the details of primary tumor with level of infiltration (T) , lymph node metastases(N) and presence of distant metastases (M). This staging is known as TNM staging.

TNM STAGING OF COLORECTAL CARCINOMA [11]

T- primary tumor

TX-primary tumor cannot be assessed

T0- No evidence of primary tumor

Tis- Carcinoma in situ; intraepithelial neoplasia or invasion into lamina propria

T1 –Tumor invades submucosa

T2-Tumor invades muscularis propria

T3- Tumor invades through muscularis propria into subserosa or into non peritonealised pericolic or perirectal tissues

T4-Tumor directly invades other organs or structures and/or perforates visceral peritoneum.

N – regional Lymphnodes

NX – Regional lymphnodes cannot be assessed

N0- No regional lymph node metastases

N1- Metastases in 1 to 3 regional lymphnodes

N2- metastases in 4 or more regional lymphnodes

M- Distant metastases

MX- Distant metastass cannot be assessed

M0- No distant metastases

M1- Distant metastases

Stage grouping:

Stage 0	Tis	N0	M0
Stage I	T1 T2	N0 N0	M0 M0
Stage II	T3 T4	N0 N0	Mo M0
Stage III	Any T Any T	N1 N2	M0 M0
Stage IV	Any T	Any N	M1

CLINICAL FEATURES:

About 5% to 12.5% of the patients are asymptomatic. [16]

The clinical symptoms depends on tumor location(right or left), stage of the disease (early or late).

- Weight loss and malaise may be the first symptoms which are often ignored by the patient.
- The right sided lesions, say tumour in the right side of colon are usually flat or polypoidal and rarely cause obstruction. These lesions remain clinically silent for a long period of time.
- Occult or massive rectal bleeding: occult bleeding diagnosed as an incidental finding in the stool serves as an indicator of colorectal carcinomas. Bleeding may be so severe to cause iron deficiency anemia thereby causing weight loss and malaise. 10.3% of patients with clinical history of rectal bleeding are found to have proven colon cancer.[17]
- In rare cases the patients presents with cardiac failure due to anemia.[17]
- Altered bowel habits:
    - Seen in 22% to 58% of patients.
    - Prevalent in left sided lesions.

- The changes are minimal at the early stages and as the tumor increases in size and encircles the entire colonic wall it causes constipation, obstipation and a sense of incomplete rectal emptying.

- Abdominal pain is the presenting symptom in some of the cases. The pain mostly occurs in cancers invading the serosa and adjacent structures. Rarely large masses, completely obstructing the lumen also cause pain. Lower abdominal pain mostly occurs in patients with caecal or ascending colon lesions. The tumors of ileocaecal valve causes obstruction of appendiceal lumen causing appendicitis..[18]

TUMOR MARKERS:

These are the diagnostic biomarkers that helps in identification of recurrent disease:

1] Carcinoembryonic antigen (CEA)

2] Cytokeratins

3] Tumor M2-PK

4] Circulating nucleic acids

1] The well known soluble diagnostic biomarker of colorectal carcinomas is ‘CARCINOEMBRYONIC ANTIGEN’, shortly called CEA. This antigen serves as a cell adhesion molecule in early fetal life, the production of which ceases in adult hood. An increased level of CEA is noted in healthy smokers and also in many benign and malignant conditions. The benign lesions include pancreatitis, liver cirrhosis, ulcerative colitis, diabetes mellitus type 1 and type 2. The various malignant lesions showing increased levels of CEA are colorectal cancer, gastric cancer, pancreatic cancer, lung cancer and breast cancer. [19]

2] The potent rival of CEA in predicting recurrence of colorectal carcinomas is circulating cytokeratins. In a study done by Fernandez and his colleagues it was found to be better than CEA. They examined 120 colorectal carcinoma patients, and an elevated cytokeratin level was found in 48% of them with recurrent disease whereas CEA was increased in only 30% of recurrence cases.[20]

3] Tumour M2-PK:

This is an enzymatic marker that can be identified in the stool. This is a dimeric form of the glycolytic pyruvate kinase isoenzyme. There is an upregulation of this enzyme in all proliferating cells. This test has a sensitivity of 91% and specificity of 79% respectively. [21]

4] Circulating Nucleic acids:

Circulating tumor DNA and mRNA in blood of patients with colorectal cancer serves as a marker for recurrence. In a study by Deihl . F it was concluded that circulating levels of detectable tumor DNA in patients' blood during the first post operative visit accurately predicted the recurrence of tumor in the future.[22]

MOLECULAR GENETICS OF COLORECTAL CARCINOMA

Colorectal carcinomas can be hereditary or sporadic. Lynch and Lynch in 1998 published a classification of hereditary disorders predisposing to colorectal carcinomas. They have not only classified the different cancer syndrome but also explained their pattern of inheritance and associated gene mutation, polyp information, other non-cancerous characteristic of the syndrome. [23]

According to them the predisposing syndromes are:

- Familial adenomatous polyposis
- Attenuated familial adenomatous polyposis
- 11307 K mutation in Ashkenazi Jews
- Juvenile polyposis coli

- Hereditary adenomatous polyposis
- Peutz – Jeghers syndrome
- Familial adenomatous polyposis
- Familial ulcerative colitis and Crohn's disease.

Most of them are autosomal dominant except Familial colorectal cancer , Familial ulcerative colitis and Crohn's disease.

Hereditary cancer syndromes are broadly divided into Polyposis and Non polyposis syndromes. Polyposis syndromes includes adenomatous and hamartomatous polyposis. Non polyposis syndromes include lynch syndrome, Muir – Torre syndrome and Turcot syndrome.

HAMARTOMATOUS POLYPOSIS SYNDROME

Hamartomatous polyposis syndrome includes Peutz – Jeghers polyps and Juvenile polyposis.

PEUTZ –JEGHERS POLYPOSIS:

These hamartomatous polyps are characteristically seen in stomach, small intestine and colon. They are architecturally different resembling a branching tree due to the

infiltration of muscularis mucosa into the mucosa of the polyp. The patients have pigmentation in the lips and oral cavity. This is due to the mutation in the gene coding for serine- threonine kinase on chromosome 19p13.3. [24]

JUVENILE POLYPOSIS:

Most commonly it occurs in children and is characterised by diffuse presence of 10 or more hamartomatous polyps throughout the gastrointestinal tract. Mostly they are benign and have mutation in the gene coding for tyrosine –phosphate protein. (PTEN) [23]

ADENOMATOUS POLYPOSIS SYNDROME

The adenomatous polyposis syndrome includes Familial Adenomatous Polyposis and Attenuated Familial Polyposis Syndromes.

FAMILIAL ADENOMATOUS POLYPOSIS

Familial adenomatous polyposis is an autosomal dominant disorder characterised by the presence of numerous colorectal adenomas during their teens. Atleast 100

polyps are neccassary for a diagnosis of classic Familial Adenomatous Polyposis. Sometimes thousands of polyps can be present. It is caused by the germ line mutation of adenomatous polyposis coli (APC) gene .

Individuals who are born with one mutant APC gene develop Familial adenomatous polyposis and invariably one or more of these polyps undergo malignant transformation resulting in colorectal carcinoma. APC gene is a tumour suppressor gene, so both copies of it must be lost for a tumour to develop.

Adenomatous polyposis coli gene (APC) is located in chromosome 5q21-22. This is a 120 kb gene with 21 exons in which 7 are alternatively expressed. The main function of this gene is to down regulate the growth promoting signals. APC gene shows high expression in CNS, although found in all other tissues. The product of APC gene, ‘APC protein’ is a negative regulator of Wnt signalling pathway. There are several domains in the gene that acts as binding and degradation sites for beta catenin, among which the one at the carboxy terminal end by mediating the phosphorylation of GSK3b forms a stabilising complex between GSK3b and APC. This in turn mediates phosphorylation of conduct / axin that recruits beta catenin and targeting its degradation through APC dependent ubiquitin – proteasome pathway.

Normally Wnt signalling releases beta catenin from its inhibitory complex (GSK3b – axin), causing its accumulation in the cytoplasm. Beta catenin in the cytoplasm binds to microtubules and also interacts with E-cadherin (cell adhesion potein). Rest of the free beta catenin moves to the nucleus and binds to TCF/LEF transcription factor family activating c-myc oncogene and cyclin D1 protein , whose uncontrolled activation in the absence of APC gene regulation culminates in carcinogenesis.

Other genetic mutations associated are TP 53, loss of 17p, 18 and 22 alleles. Also k-ras mutation causes cyclin D1 transcription and promotes carcinogenesis. [25]

Colonic cancer develop in 100% of untreated FAP patients before the age of 30 years; hence a prophylactic colectomy is the standard therapy for individuals with APC gene mutation. [26]

FAP is also associated with other extra – intestinal manifestations like development of Gardner syndrome and Turcot syndrome. Gardner syndrome is characterised by multiple adenomas, osteomas, epidermal cyst, desmoid tumors, thyroid tumors and dental abnormalities. Turcot syndrome is characterised by intestinal adenoma and tumors of central nervous system like meduloblastoma and glioblastoma. [26]

ATTENUATED FAMILIAL POLYPOSIS SYNDROMES

Interestingly correlations have been observed between the location of APC gene mutation and the phenotype. Attenuated APC (AAPC) occurs when there is mutation in the first and last third part of the gene. It is characterised by late onset with less than 100 polyps and have a prevalence for fundic gland polyps. When mutation occurs in the central region, in the codon1309 – there is a severe phenotype characterised by about 1000 polyps at young age, whereas mutation at codon 386 or 1465 only mildly interferes with APC gene function. [27]

LYNCH SYNDROME:

Also called hereditary non polyposis colorectal cancer [HNPCC] is an autosomal dominant condition and is characterised the increased risk of colorectal cancer along with carcinomas of endometrium, ovary, stomach, urinary tract and glioblastoma multiforme of brain. Lynch patients have an early onset of right sided colorectal carcinoma and is associated with synchronous and metachronous cancers. Amsterdam criteria (Table 1) were established in 1991 for the diagnosis of lynch syndrome. [28]

TABLE 1 AMSTERDAM CRITERIA 1991

Three or more relatives with colorectal cancer plus all of the following
• One affected person is a first-degree relative of the other two affected persons
• At least two successive generations should be affected
• At least one cancer should be diagnosed before age 50

Later Amsterdam criteria 1 were revised in 1999. Table 2 shows revised Amsterdam II criteria [29]

TABLE 2

Amsterdam II criteria (Revised International collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (HNPCC) Criteria 1999

There should be at least three relatives with colorectal cancer or with a lynch syndrome associated cancer plus all of the following

- One relative should be a first degree relative of the other two
- Atleast two successive generations should be affected
- Atleast one tumor should be diagnosed before the age of 50 yrs
- FAP should be excluded in CRC case if any
- Tumors should be verified by histopathological examination.

Genes that underlie HNPCC are the mismatch repair genes that includes MLH1, MSH2, MSH6 and PMS2. The function of these genes is to correct the base –base mismatches that occur during DNA replication, failure of this leads to insertion or deletion of short repetitive sequences in the DNA coding regions causing microsatellite instability. [30]

Muir –torre syndrome:

A type of Lynch Syndrome with sebaceous tumours and are caused mainly by MSH2 mutation. [26]

Turcot syndrome:

This syndrome is characterised by the presence of medulloblastoma , glioblastoma, colorectal cancers and rarely polyposis . Patients with medulloblastoma has APC gene defect and those with glioblastoma has primarily PMS 2and MLH1 defect [26]

About 90% of colorectal carcinomas associated with HNPCC show microsatellite instability with predominant mutation in either MLH1 or MSH2 or both. [31]

GENETIC PATHWAYS OF COLORECTAL CANCER

The various genetic pathways leading to colorectal carcinomas include; [32]

1) Adenoma – Carcinoma sequence

2) Serrated neoplasia pathway

3) Microsatellite instability pathway and mutation of MMR genes.

Adenoma – Carcinoma sequence

This pathway is also called suppressor pathway due to the inactivation of tumor suppressor genes in the carcinogenesis. The various genes involved are APC, TP53, SMAD4 located on chromosomes 5q, 17p and 18q respectively.

Activation of k-ras oncogene and deletion of DCC [deleted in colorectal cancer] located on 18q are other proposed alterations in this pathway.

Serrated neoplasia pathway:

Hyperplastic polyps progress to serrated adenoma and later to carcinoma. These tumors have a serrated epithelial lining. Tatayema et al proposed that the molecular

basis of these tumors may be the decreased expression of CD 95 of apoptosis. About 15% show defect in Wnt signaling pathway. Other mechanisms suggested are BRAF and K-ras mutation. [33]

Microsatellite instability and MMR pathway:

The hall mark of HNPCC is microsatellite instability. Microsatellites are simple repetitive DNA sequences in a genome and are composed of one or more base pair units that may be repeated several times. During DNA replication, errors can occur in their repetitive subunits resulting in contraction or elongation of a microsatellite sequence, a process described as microsatellite instability (MSI). This is important when MSI are in the critical areas of the gene responsible for cell growth regulation. Mismatch repair genes play a crucial role in detecting and correcting these types of errors. Failure to correct these errors due to defect in mismatch repair genes can lead to frame shift mutations with loss of normal function of these genes and development of tumour. [34]

There are several mismatch repair genes. The most important genes include MSH2 (Mut S homologue 2), MSH6 (Mut S homologue 6), MLH1 (Mut L homologue 1), MSH3 (Mut S homologue 3) and PMS2 (Postmeiotic segregation 2). The MSH2 protein recognises and binds to the mismatched sequence. If a single base pair

mismatch is recognised it forms a heterodimeric complex with MSH6 and if there is a larger 2 to 8 nucleotide insertion or deletion it combines with MSH3. A second heterodimeric complex of MLH1 and PMS2 is recruited which subsequently directs the remaining set of genes to excise the mismatched nucleotide. [35]

MMR genes are expressed in all adult tissues but are most prominent in the epithelium of gastrointestinal tract, testis, ovary and is highly expressed in the replication zone.

MSI is seen in nearly all tumours of HNPCC and about 15% of sporadic cancers. HNPCC is transmitted as an autosomal dominant trait and shows germline mutation in one or more of the mismatch repair genes. Mutations of MLH1 and MSH2 are more common and they occur in exon 16 and exon 12 of the genes respectively. [36] Another 12% of colorectal carcinomas represent non-inherited form of DNA mismatch repair gene inactivation due to methylation of the promoter of the MLH1 gene, which can cause defect in its function.[37]

MSI- tumors, mostly arises in the right side colon which has a poorly differentiated mucinous phenotype and a female preponderance , but it has a good prognosis.

METHODS OF TESTING MICROSATELLITE INSTABILITY

Microsatellite instability is due to insertions or deletions of sequences in short tandem repetitive sequences in DNA. The only method for identifying these sequences is by PCR amplification of the tumour DNA with the five markers recommended by National Cancer Institute conference on MSI.

MSI testing:

MSI testing was first performed by Peltomaki et al who was the first to document the genetic basis of HNPCC. [38]

Extraction of DNA:

To do a MSI analysis, DNA is obtained from the tumour and adjacent normal tissues by proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation.

Selection of markers:

A standard panel consisting of 5 microsatellite markers as suggested by National Cancer Institute is used. This includes BAT26, BAT40, D2S123, D5S346 and D17S250. These are used as primers for the subsequent PCR reaction.

The reaction mixture consisting of template DNA, Taq polymerase and the primers are subjected to 35 cycles of PCR. [39]

The PCR products then undergo PAGE electrophoresis. The alterations in microsatellites are detected by changes in the length of DNA strands when compared to adjacent normal tissue. MSI is scored by the occurrence of novel bands in the tumour DNA or similar shift in all bands with a retained normal band pattern.[40]

Based on the electrophoresis pattern, the instability is categorised as follows:[40]
MSI-H (high) – If 2 or more markers showed instability.

MSI-L (low) - If only one marker showed instability.

MSS- When there are no alterations in any of the marker.

In another study by Maingold et al [41], when there is instability in only one of the marker, an additional panel of 5 markers namely BAT 40, D10S197, D13S153, MYCL1 and D18S58 were subjected to PCR with these tissues and the results are graded as:

MSI-L – Instability in one or two of the total 10 markers.

MSI-H – Instability in 3 or more of the total 10 markers

Microsatellite instability, though a signature feature of lynch syndrome is also seen in 15% of sporadic colorectal carcinomas. The microsatellite instability in these cases is mostly caused by methylation induced silencing of MLH1 gene.MSI-L tumours do not show the characteristic histopathological features of MSI-H tumours and they are not associated with germ line mutations in MMR genes. These cases are due to non mutational down regulation of MSH-3. [42] These individuals were selected for MSI testing based on Amsterdam criteria. Now the recently used criteria is revised Bethesda guidelines: [43]

TABLE 3

1. Patient diagnosed as colorectal cancer before the age of 50 years
2. Presence of synchronous or metachronous colorectal or other HNPCC related tumors (stomach, urinary bladder, ureter and renal pelvis, biliary tract, brain(glioblastomas), sebaceous gland adenomas, keratoacanthomas and small bowel cancer, regardless of age.
3. colorectal cancer diagnosed before 60 years of age with morphology suggestive of MSI-H (including the presence of tumor infiltrating lymphocytes, Crohn – like lymphocytic reaction, mucinous or signet ring cell differentiation, or medullary growth pattern).
4. CRC in a patient with 1 or more first degree relatives with CRC or other HNPCC related tumors(one of the cancers must be diagnosed before 50 years of age, and adenomas must be diagnosed before 40 years of age.)
5. CRC in a patient with 2 or more relatives having CRC/other HNPCC related tumours, regardless of age.

DETECTION OF MISMATCH REPAIR GENE MUTATIONS

As said earlier, loss of function of several mismatch repair genes such as MSH2, MLH1, PMS2 and MSH 6 plays a causal role in colorectal carcinomas particularly in those showing MSI-H instability. As MSI testing is labour intensive and cumbersome ways for detection of loss of MMR protein expression in tumour tissue came into existence.

The MMR protein expression can be identified by immunohistochemistry. [44] The principle behind this method is antigen antibody reaction. The blocks of resection specimen with the tumour tissue is taken and then 5 microns thick sections were cut and taken in a poly-l- lysine coated slide. Sections were incubated overnight at 56 degree C. The slides are then dewaxed and dehydrated in graded alcohol. Rehydration is done finally with distilled water. Antigen retrieval is done in a specific method with a buffer followed by cooling in room temperature. After blocking endogenous peroxidise activity, they are treated with monoclonal antibody for 1 hour and secondary antibody is added followed by chromogen which imparts brown colour to the nucleus of the cells that had taken the primary and conjugated secondary antibody.

Mutations have been identified in all the 19 exons of MLH1 and 16 exons of MSH2. These genes also have variants based on the type of mutations or genetic alterations and are hence classified as follows: [45]

1. Pathogenic mutation – generally frameshifts, nonsense and splice mutations

2. Probable pathogenic mutation – nonconservative aminoacid changes.

3. Probable polymorphisms – generally conservative changes, also observed in controls

4. Definite polymorphism- synonymous variants.

Mutations in these genes result in lack of expression of these proteins in the tissue which can be detected by immunohistochemical marker study using antibodies against these proteins.

Apart from the immunohistochemical staining for MMRproteins, there are other means of detection of the mutations in these genes which includes:

- Genomic sequencing
- In vitro synthesised protein assay
- DNA structure techniques.

GENOMIC SEQUENCING:

This is one of the most widely used methods in affluent centres. In this test all the 35 coding exons (16 exons of MSH 2 and 19 exons of MLH 1) are tested. The necessary DNA is amplified from peripheral blood leucocytes using a PCR reaction. PCR reaction is followed by an electrophoresis of end products.

Yaping Wang [46] and his colleagues did genomic sequencing to look for large deletions in the two genes MLH1 and MSH2 based on multiplex PCR using 6 pairs of primers encompassing both the genes. After a multiplex PCR, the analysis of end products was done using an agarose gel electrophoresis, visualised by ethidium bromide staining. As a result, in a total of 180 patients large deletions were seen in 19 cases.

IN VITRO SYNTHESISED PROTEIN ASSAY:

In this technique an in vitro system is used where transcription and translation of a PCR product is done which is obtained from several exons of the MLH1 and MSH2 genes. For this method mRNA is required for the production of cDNA . With this method several mutations such as frame shifts, splicing, out of frame deletions or insertions can be detected. [45]

DNA STRUCTURE TECHNIQUES:

These techniques are based on the structural changes in DNA molecule as a result of mutation. [45]

The various techniques are:

- Denaturing gradient gel electrophoresis
- Single strand conformational polymorphism
- Protein truncation test
- Heteroduplex analysis
- Two dimensional DNA typing

Out of the above techniques Farrington et al [47] found that genomic sequencing had 80% sensitivity compared to other techniques.

NEED FOR MMR TESTING

There is a need for the identification of loss of expression of mismatch repair proteins in the tumour due to various reasons.

- MMR protein loss correlates with microsatellite instable tumours that too with those showing high grade of instability. [33]
- MSI tumours are often associated with multiple synchronous and metachronous cancers that formed a basis for Bethesda criteria.

  Horri et al [48] found that there is 89% rate of MSI in 38 tumours having multiple malignancies.
- Immunohistochemical pattern of MLH1 and MSH2 expression is observed to correlate with certain histopathological characters.

In general microsatellite instability tumours have been associated with younger age at onset, more female incidence, increased involvement of proximal colon, large size, expanding pattern of growth, signet ring / mucinous / medullary morphology , peritumoral Crohn's like lymphocytic response and intratumoral lymphocytic infiltration. [38]

Giovanni Lanza [49]and his colleagues found that the immunohistochemical expression of MLH1 and MSH2 is significantly related to above histopathological

characters. In their study they evaluated immunohistochemical expression of MLH1 and MSH2 protein in 132cases of MSI and 150 microsatellite stable (MSS) colorectal adenocarcinomas. Loss of MLH1 or MSH2 expression was detected in 120 cases of MSI carcinomas, whereas all MSS tumours showed normal expression of both proteins. They also reported that MSI-H tumors,which lacked the expression of MLH1/MSH2 showed significant correlation to various clinical and histopathological variables. The tumors with positive expression of MLH1 and MSH2 were conventional adenocarcinomas of well or moderately differentiated grade . Most of them were p53 positive, size less than 7cm in diameter and located in the distal colon. Whereas the tumors that lack these MMR proteins were mostly poorly differentiated and proximal in location. In addition, MLH1-negative carcinomas were less common among patients with Hereditary Non Polyposis Colorectal Cancer (HNPCC) or suspected HNPCC and in the group of patients aged <50 years.

The clinical uses of MSI testing are:

- Microsatellite instability testing helps us to classify familial cases of colorectal carcinomas. Valle and his colleagues [50] gathered families of colorectal cancers including both MSI and MSS. They found that MSI colorectal cancer families had a younger age of onset and proximal colon

distribution and about 60% of them are significantly linked to a mutation in DNA mismatch repair gene.

- MSI cases have a survival advantage in spite of their poor differentiation in histology. This is attributed to intratumoral and peritumoral lymphocytic infiltration. Kriesten M.Drescher [51] and his men suggested that the defect in MMR proteins may be a reason for the increased lymphocytic infiltration. As a result of mutations in the MMR genes and deletions /insertions in the microsatellites, the tumour has a lot of altered proteins unique to itself. These new peptides are immunogenic and are recognised as foreign by the immune system. As a result there is infiltration by lymphocytes, particularly CD8+ T lymphocytes.

- Another significant implication of loss of mismatch repair proteins is that these tumors are relatively insensitive to 5-flurouracil chemotherapy. Kim and his colleagues [52] stated that normally , DNA MMR system is responsible for signalling cell death when there is significant toxic DNA damage, whereas in these CRCs as there is loss of MMR genes function, these tumours are not susceptible to the damage caused by 5 fluorouracil, and are hence selectively resistant to 5FU based chemotherapeutic regimens.

Vijay pandey and his co-men assessed mismatch repair protein expression in 46 patients with simultaneous detection of microsatellite instability using the five Bethesda markers. Assessment of microsatellite instability in colorectal carcinoma at an Indian center indicated that the incidence of MSI was similar to that of the west, despite lower incidence of colorectal cancers and predominance of rectosigmoid tumors in the Indian population. [39]

Mariann christensen [40] and his colleagues compared IHC, MSI analysis and genomic sequencing of MLH1 and MSH2 genes in colorectal carcinomas, in patients either strictly meeting Amsterdam criteria or are suspected to be a HNPCC family. They concluded that IHC is comparatively more cost effective and can be used as a first screening test for detecting HNPCC families.

MATERIALS AND METHODS

The cases diagnosed as colorectal carcinomas from colonic resection specimens during the period 2008 to 2011 in the department of pathology, PSGIMSR were included in the study. The case selection was done by systematic random sampling. The colonoscopic biopsy specimens were rejected from the study.

The clinical details like age, sex of the patients and other gross findings necessary such as site, size of the tumor were taken from the requisition slips. The representative paraffin blocks and H&E slides were retrieved from the archives of pathology. Paraffin blocks of the slides with high tumor density were chosen for the study. 4μ thick sections were made from the chosen blocks for routine hematoxylin and eosin staining. Then the cases were screened for histological type of the tumor and grade of differentiation. Tumor status and lymph node involvement status, as per the TNM staging were analyzed and noted in the master chart.

HAEMATOXYLIN AND EOSIN STAINING

REAGENTS REQUIRED:

1. Harris haematoxylin

2. Eosin

PROCEDURE:[53]

1. Deparafinisation: done with xylene

2. Hydration: hydration is done through subjecting the sections to immersion in graded alcohols followed by bringing into water.

3. Haematoxylin staining: sections were stained with harris hematoxylin stain for 10 minutes.

4. Wash the haematoxylin stained slide in running tap water until sections become blue (5 minutes)

5. Differentiation: done with 1% acid alcohol which is a mixture of 1% hydrochloric acid in 70% alcohol - 10 seconds

6. Wash with tap water for 10 minutes

7. Bluing by immersing the slide in ammonia water followed by washing in tap water for 5 minutes.

8. Eosin staining: stain in eosin for 10 – 15 minutes.

9. Wash the stained slide in running tap water for 3 minutes

10. Dehydration through graded alcohols

11. Clearing

12. Mounting.

After haematoxylin and eosin staining, the slides were reviewed once again for adequacy and sections are taken from the respective blocks on a poly-l-lysine coated slide for immunohistochemical staining.

IMMUNO HISTOCHEMICAL STAINING

Tissue blocks of normal colon sent for non – neoplastic lesions were taken as controls. The blocks were then cut to 5µ thick sections and taken on a Poly-L-lysine coated slide to serve as positive control for MLH1 and MSH2 antibody.

Immunohistochemistry for detection of expression of MLH1 and MSH2 was performed using the supersensitive HRP-polymer detection system with the appropriate control. The procedure executed is described below:[54]

ANTIBODIES USED:1] MLH 1(novocastra , clone ES05)

2] MSH 2 (novocastra , clone 25D12)

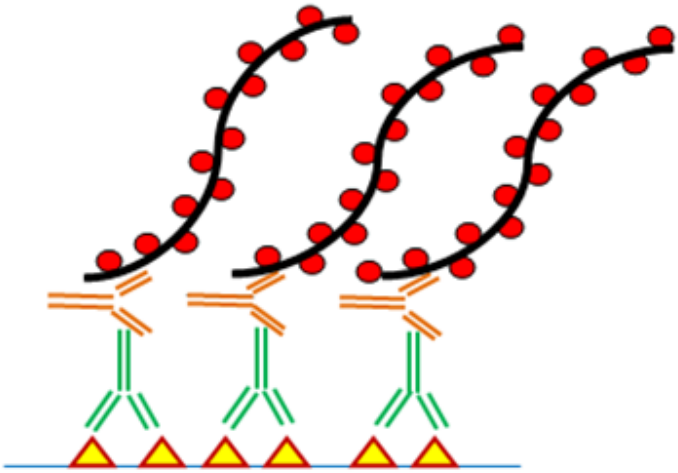
PRINCIPLE OF THE TEST: In an immunohistochemical reaction, the specific antigen present in the cells and tissues was detected in a two stage process that includes:

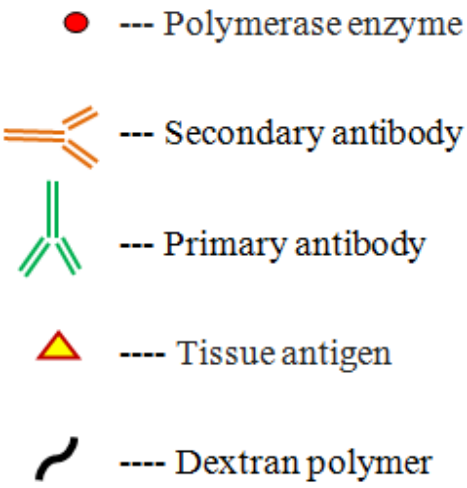
1) The binding of the primary antibody to its specific epitope in the tissues.

2) Detection of this primary bound antibody using a dextran polymer bound secondary antibody in a calorimetric reaction involving a chromogen.

In this method, primary antibody to the specific antigen is first added and is then followed by the addition of a dextran polymer linked to multiple conjugated secondary antibodies and horse raddish peroxidase enzyme. This multiple secondary antibodies bound to the primary antibody and the signal is amplified by the use of a suitable chromogen 3, 3'diaminobenzidine tetra hydrochloride (DAB)

Fig 1: Principle of Immunohistochemistry





Antigen retrieval:[54]

This is a process to unmask the epitopes of the specific antigens that are masked by cross linking action of formalin during routine processing. There are various methods for antigen retrieval. The methods include,

1) Pressure cooker method

2) Microwave method

3) Proteolytic digestion method

Of the above, the pressure cooker method is used for the present study. Here, the tissue is exposed to the additive effects of both heat and pressure thereby bringing out the full antigenicity. After dewaxing and hydrating in graded alcohols, the slides were subjected to antigen retrieval in a pressure cooker for 10 minutes with EDTA buffer at pH 9.

REAGENTS USED:

- EDTA buffer at pH 9. [ethylene diamine tetra acetic acid]
- 3% hydrogen peroxide (H2O2) in distilled water - To block endogenous peroxidase activity in order to prevent nonspecific background staining

- 0.01M Phosphate buffered saline (PBS) with a pH value of 7.6. It was prepared by dissolving the following substances in 1000 ml of distilled water.

  1. Na2HPO4 Dibasic sodium phosphate, anhydrate 17.5g

  2. KH2PO4 Monobasic potassium phosphate, anhydrous 2.5g

  3. NaCl Sodium chloride 17.0g

- Blocking reagent- casein in PBS with 15mM sodium azide. This was used to block non specific protein binding.

- Primary antibodies against MLH1 and MSH2 antigen. As the antigens were in a concentrated form, they are diluted as specified in the user manual as follows, before using

  1. MLH 1 antibody is diluted with PBS in a ratio of 1:100

  2. MSH2 antibody is diluted with PBS in a ratio of 1:50

- Poly HRP reagent- anti-mouse and anti-rabbit IgG complex linked to Horse radish peroxidase enzyme.

- DAB (3, 3'Diamino Benzidine tetra hydrochloride) - Chromogen.

It offers great sensitivity as an HRP calorimetric chromogen and provides insoluble permanent coarse brown precipitate.

- Harris hematoxylin as counter stain.
- DPX (Distrene dibutyl phthalate Xylene) - Mountant.

PROCEDURE:

1. Immunohistochemical Staining with the two specific antibodies were done as follows
    - Slides were dewaxed .
    - Dewaxed slides were hydrated using graded alcohol.
    - Antigen retrieval: using EDTA buffer at pH 9.0 in a pressure cooker for 10 minutes.
    - Fast cooling under tap water.
    - Washed in PBS buffer at pH 7.6 for 5 minutes
    - After wiping off excess PBS buffer the slides were immersed in 0.3% H2O2 for 20 minutes to block endogenous peroxidase activity.

- Washed in PBS buffer thrice,5 minutes each
- Slides were incubated in blocking solution for 10 minutes to block non-specific protein binding.
- Washed in PBS buffer thrice , each 5 minutes.
- Slides were incubated with diluted MLH 1 primary antibody for 1 Hr.
- To enhance the signal intensity, the sections were put in superenhancer for 30 minutes.
- Washed in PBS buffer thrice, each for 5 minutes.
- Horse radish peroxidase polymer reagent was added to the slide and incubated for 30 minutes.
- Washed in PBS buffer thrice, each for 5 minutes.
- Chromogen Diamino Benzidine (DAB) was applied for 8 minutes.
- Washed in PBS buffer thrice, each 5 minutes.
- Sections were counter stained with Harris hematoxylin for 1 minute.
- Washed in tap water.
- Sections were cleared in Xylene and mounted with DPX mountant.

The same procedure is repeated in another section from the same block using the second primary antibody MSH2.

The stained sections were screened to analyze the expression of the two antigens, MLH 1 and MSH2. Apart from the control sections taken from the specimen sent for non-neoplastic lesion, the cells in the crypt epithelium and lymphocytes in the normal mucosa of the respective sections were also be used as positive control. The presence of these two antigens are expressed as brown colour nuclear staining.

Slides were considered positive when they show a brisk nuclear positivity. Less than 5% of cells showing positivity were considered negative.[44]

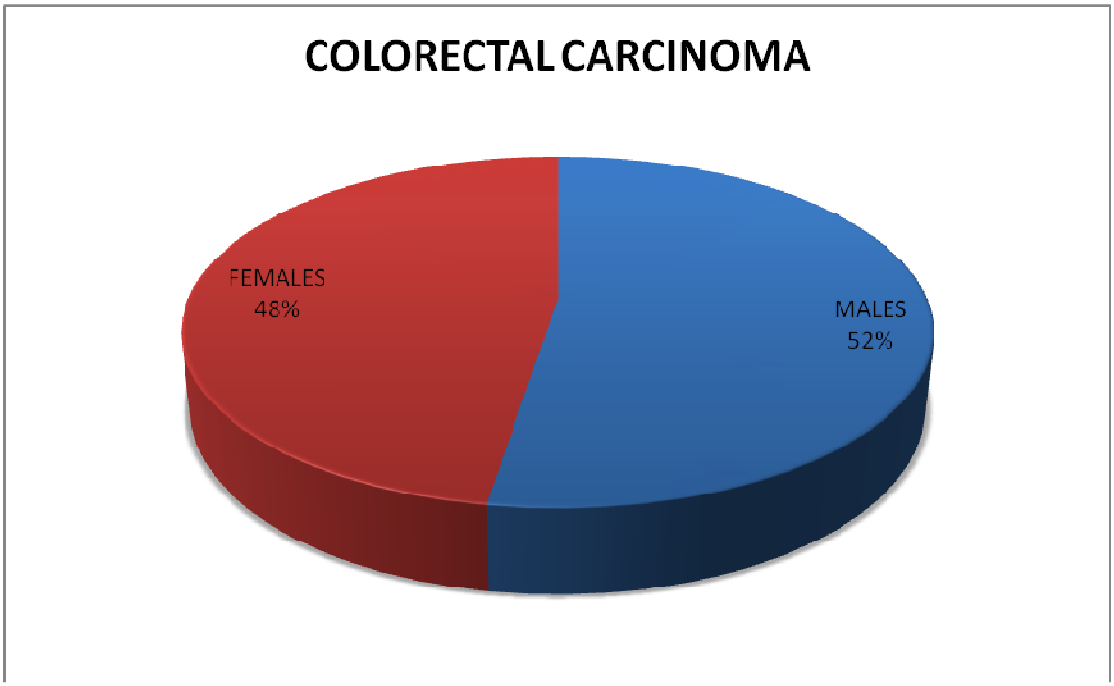
RESULTS

There were 40 cases of colorectal carcinoma, of these 21 cases (52%) were males and 19 (47.5%) were females (Graph 1). Male to female ratio was 1.1: 1. The age range was between 33 and 85 years with a mean age of 58.9 years. Maximum number of cases were between the age group of 41 to 80 years (Table4 & Graph2). The youngest patient was a 33 year old female.

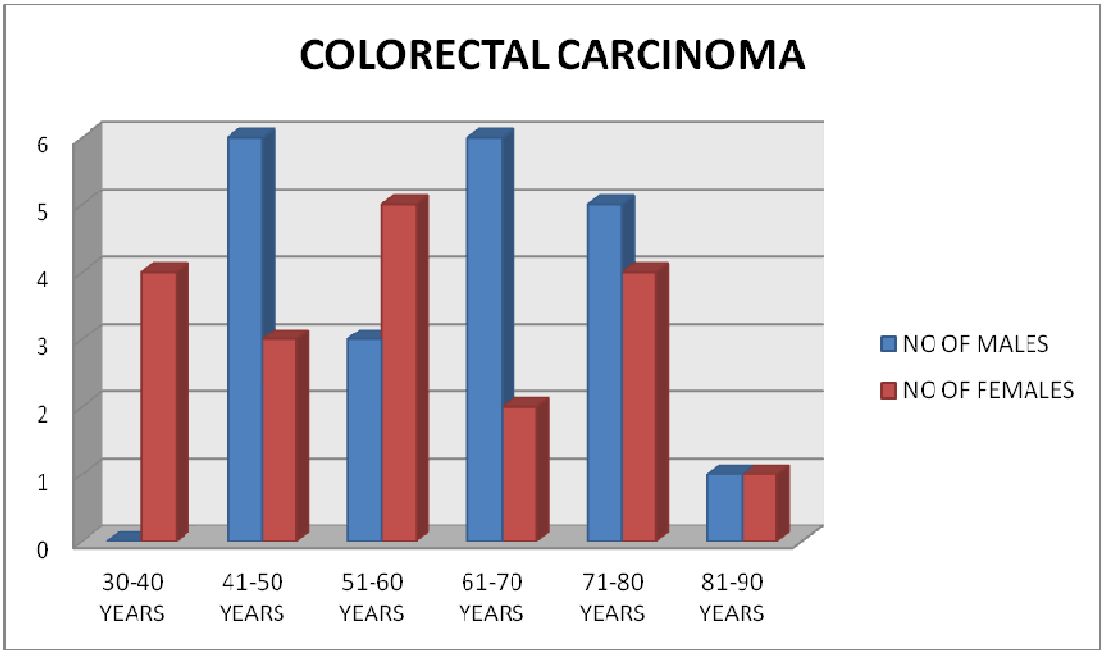
Table 4: Age distribution of the cases

Age (years)	No of cases	No of males	No of females
30-40	04	00	04
41-50	09	06	03
51-60	08	03	05
61-70	08	06	02
71-80	09	05	04
81-90	02	01	01
TOTAL	**40**	**21 (52%)**	**19 (47.5%)**

Graph 1: Gender distribution of cases



Graph 2: Age distribution of cases



A high proportion of the tumors were located on the distal colon. Out of 40 cases, 15 cases had tumor proximal to splenic flexure and 25 had tumor distal to splenic flexure. Maximum number of cases (12/40) were seen in rectum(30%), followed by sigmoid colon (9/40). (Table 5)

TABLE 5: Site of tumors

Site	No of cases
Caecum	04 (10%)
Ascending colon	04 (10%)
Transverse colon	07 (17.5%)
Descending colon	03 (7.5%)
Sigmoid colon	10 (25%)
Rectum	12 (30%)

The sizes of the tumors were ranging from 1.0 cm to 8.0 cm in greater dimension. 20 cases were 3.0 to 6.0 cm in size. 9 cases were less than 3.0 cm and another 9 cases were more than 6.0 cm in size. Majority of the tumors (32 cases, 80%) were conventional adenocarcinoma, 6 cases (15%) were mucinous adenocarcinoma and 2 cases (5%) were signet ring cell carcinoma. (Table 6)

TABLE 6: Histological type of the tumors

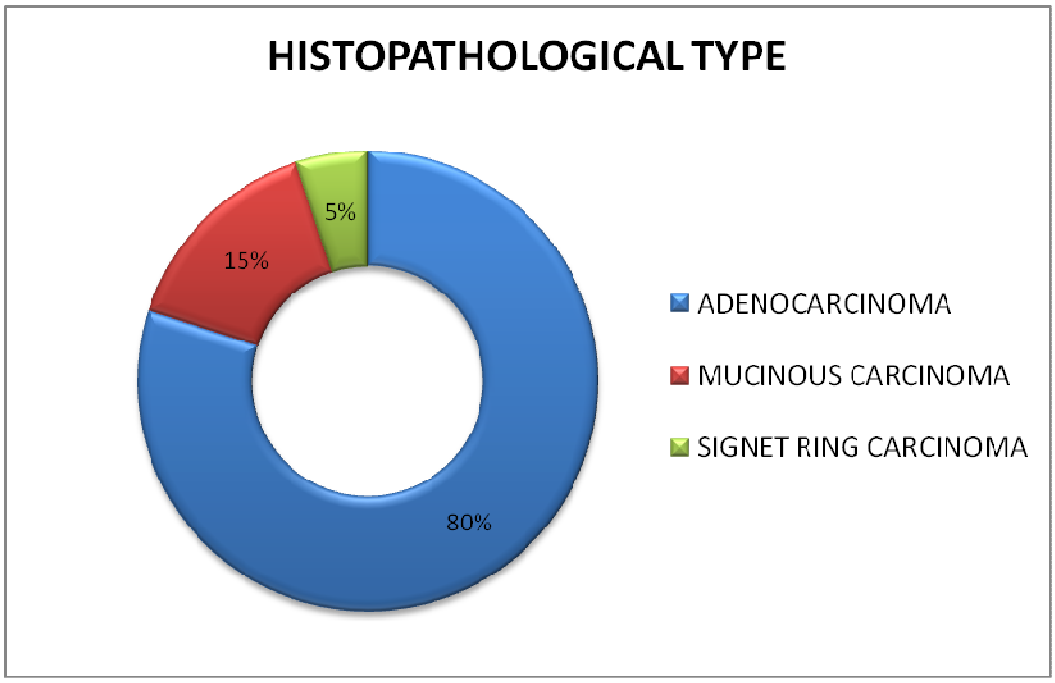
Histological type	No of cases
Adenocarcinoma	32 (80%)
Mucinous carcinoma	06 (15%)
Signet ring cell carcinoma	02 (5%)
Total	40 cases

Histopathologically, the tumors were graded into well differentiated(fig 2), moderately differentiated(fig 3) and poorly differentiated. The mucinous(fig4) and signet ring cell carcinomas(fig 5) were also considered as poorly differentiated. Majority of them (27 cases, 67%) were moderately differentiated. 9 cases (22.5%) including the other variants like mucinous and signet ring cell carcinomas were poorly differentiated. (Table 7, graph 3 and graph 4)

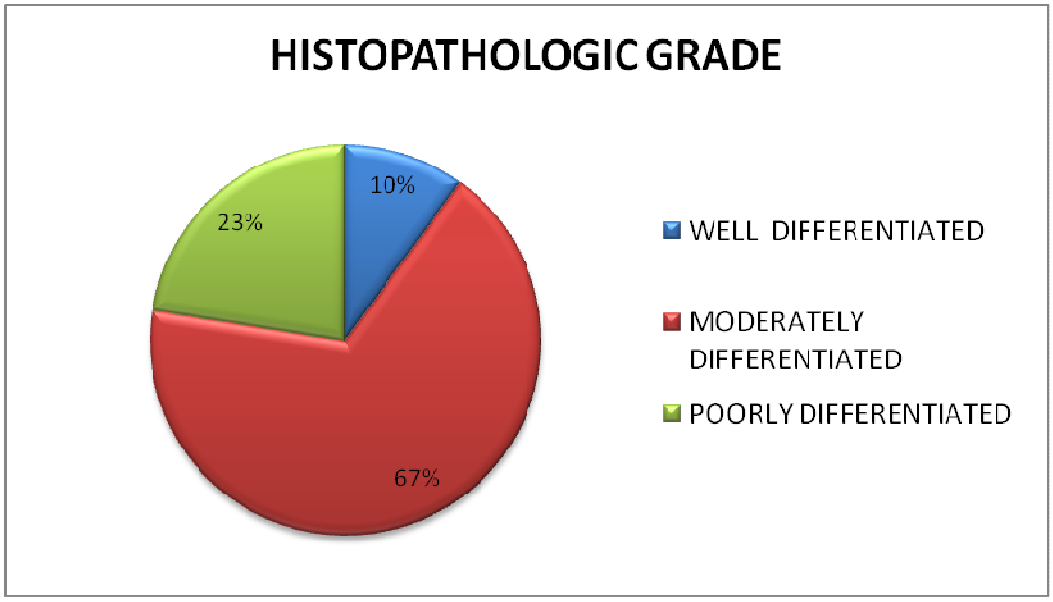
Table 7: Degree of differentiation of tumors

Histopathological grade	No of cases
Well differentiated	04 (10%)
Moderately differentiated	27 (67.5%)
Poorly differentiated	09 (22.5%)
Total	40 cases

Graph 3 : Histological type of tumors



Graph 4: Degree of differentiation of tumors



Majority of the cases (70%) were in stage 3 and 25% of cases were in T1 stage. Lymph node metastases were seen in eleven cases. (table 8&9)

Table 8: 'T' status (stage) of tumors

Tumor stage (T)	No of cases
T0	00
T1	01(2.5%)
T2	10 (25%)
T3	28(70%)
T4	01(2.5%)
Total	40 cases

Table 9: 'N' status (lymph node involvement) of tumors

Lymph node involvement	No of cases
N0	29(73%)
N1	8(20%)
N2	3(7%)
Total	40 cases

IMMUNOHISTOCHEMICAL DETECTION OF MMR PROTEINS

Of the 40 cases, 12 (30%) patients had lack of expression of MSH2 protein, 4 (10%) patients had lack of both MLH1 and MSH2 protein while the remaining 24 cases (60%) showed both MLH1 and MSH2 positivity. (Table 10 , Graph 5)

Table 10: Lack of MMR protien expression in the study population.

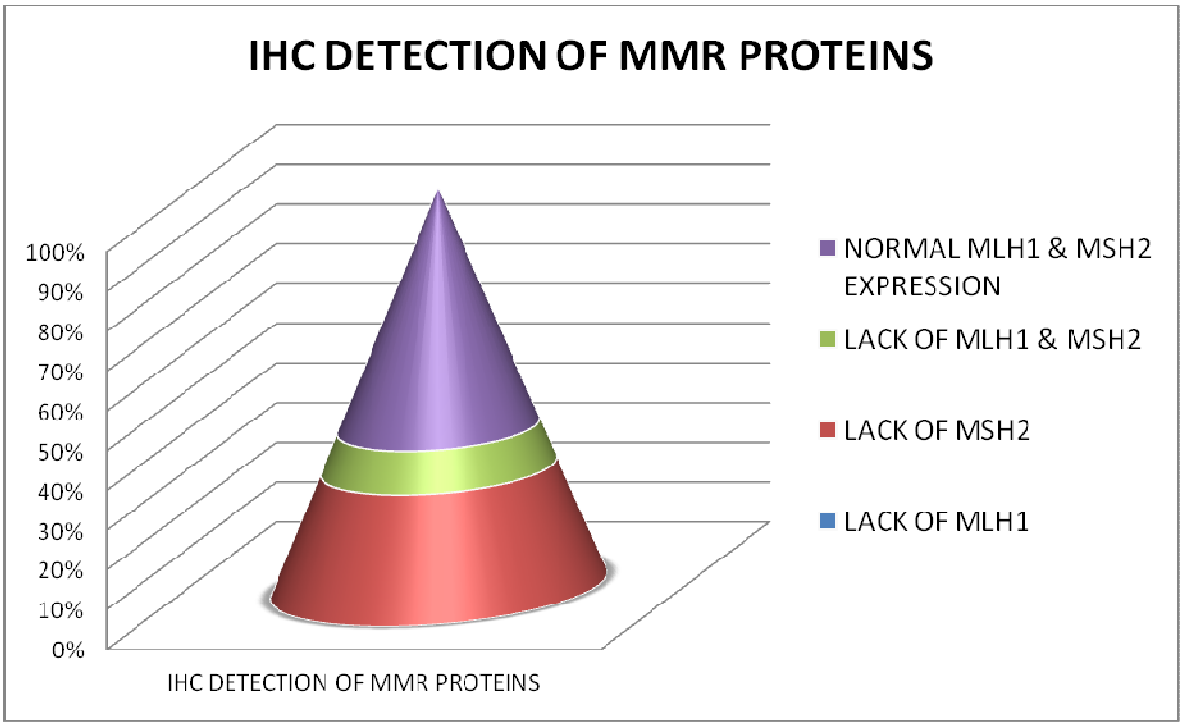
Lack of MLH1	Lack of MSH2	Lack of MLH1 & MSH2	Normal MLH1 and MSH2 expression
0	12 (30%)	4 (10%)	24 (60%)

Out 40 cases, 16 (40%) cases showed lack of expression of either MSH2 or both MLH1 and MSH2. There were no cases with lack of only MLH1 expression. Among these 16 cases there was equal distribution of males and females. (Table 11, Graph 6).

Table 11: Gender distribution in cases showing MMR protein loss

Sex	Total	Total no cases lacking MLH1/MSH2	Lack of MLH1	Lack of MSH2	Lack of MLH1 & MSH2
Males	21	8	0	7 (33%)	1 (5%)
Females	19	8	0	5 (26%)	3 (16%)

Graph 5: Lack of MMR protien expression in the study population.



Graph 6 : Gender distribution in cases showing MMR protein loss

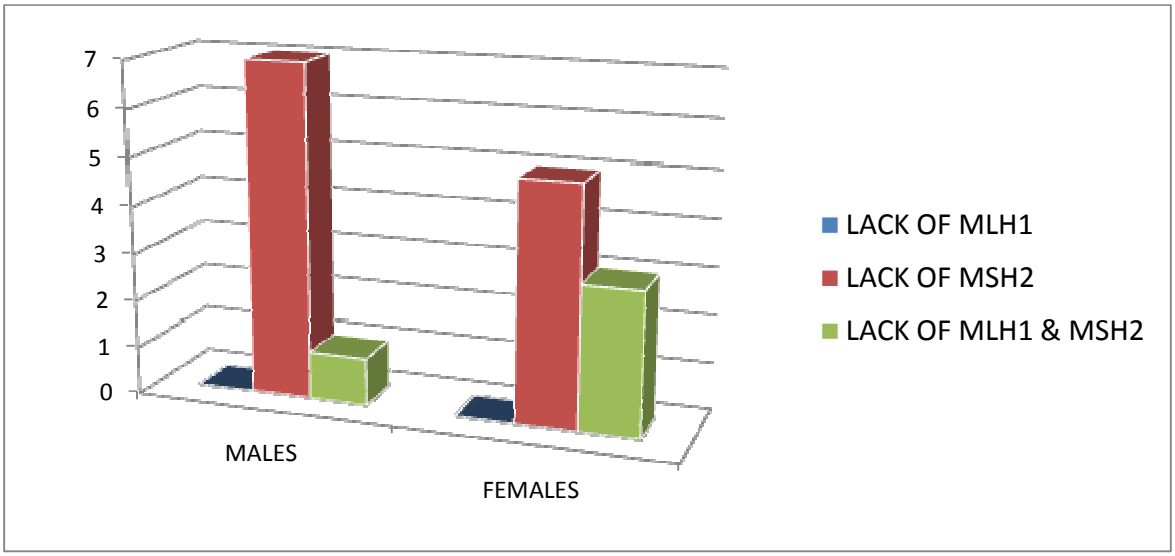


Table 12: Age distribution in cases showing lack of MMR protein expression

Age (years)	No of cases	Lack of MLH1	Lack of MSH2	Lack of MLH1 and MSH2
30-40	04	00	00	01 (25%)
41-50	09	00	04 (44%)	00
51-60	08	00	02 (25%)	00
61-70	08	00	00	01 (12.5%)
71-80	09	00	05 (55.5%)	02 (22%)
81-90	02	00	01 (50%)	00
Total	40	00	12	04

Out of 16 cases which showed loss of mismatch repair proteins, about half (50%) of the case where in their 7^{th} decade , implying that in our study population , elderly persons featured an increase in lack of expression of mismatch repair proteins. (Table 12)

Table 13: Size of tumor in correlation with MMR protein loss

Size	Total	Lack of MLH1	Lack of MSH2	Lack of MLH1 & MSH2
Less than 3 cm	5	0	3(60%)	0
3< size <6 cm	29	0	8(25%)	2(7%)
> 6cm	6	0	1(16.7%)	2(33%)

The sizes of the tumors were ranging from 1cm to 9 cm in greater dimension. Majority of the cases (29/40) were between 3cm and 6 cm and 5 cases were less than 3cm in size. Out of 5 cases with size less than 3 cm, 3 cases (60%) showed isolated negative staining for MSH2 protein. Lack of expression of both MSH2 and MLH1 was common among tumors measuring > 6 cm in size (Table 13).

In our present study 32/ 40 cases were conventional adenocarcinomas and among these only 10 cases showed alteration in expression of MLH1 / MSH2 protein. Remaining 22 cases had normal expression of MLH1/ MSH2 protein(fig 6 and fig 7) (Table 14)

Table 14: Histological type of tumor in correlation with lack of expression of MMR proteins.

Histological type	Total	Lack of MLH1	Lack of MSH2	Lack of MLH1 & MSH2
Adenocarcinoma (conventional)	32	0	6 (19%)	4 (12.5%)
Mucinous adenocarcinoma	6	0	5 (83%)	0
Signet ring cell carcinoma	2	0	1 (50%)	0

When lack of expression of MLH1 and MSH2 was correlated with tumor grade it was found to be associated with higher grade. Of the 16 cases which showed MLH1 / MSH2 alteration or both, 7 cases were moderately differentiated and remaining 7 cases were poorly differentiated (fig 8 and fig 9) .

In our study mucinous adenocarcinoma and signet ring cell carcinoma(fig 10 and fig 11)were considered as poorly differentiated and 83% of mucinous adenocarcinomas (5/6) showed lack of MSH2 expression. (Table 15)

Table: 15 : Grade of tumor in correlation with MMR protein loss

Tumor Grade	**TOTAL**	**Lack of MLH1**	**Lack of MSH2**	**Lack of MLH1 & MSH2**
Well differentiated	4	0	2 (50%)	0
Moderately differentiated	27	0	4 (15%)	3(11%)
Poorly Differentiated	9	0	6 (67%)	1(11%)

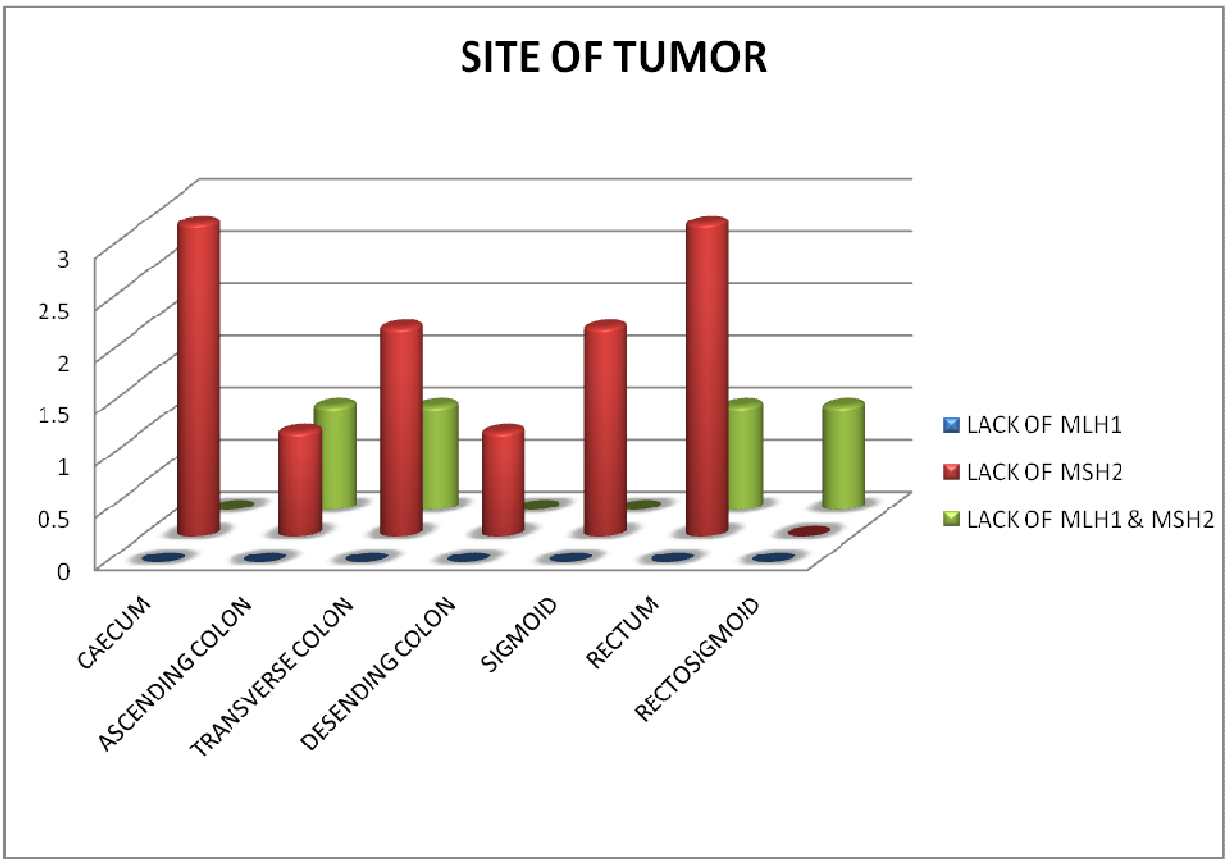
While correlating lack of MLH1 and MSH2 expression with site of occurrence, it was found to be associated mostly with rectal origin (77%) and it showed isolated loss of MSH2 in 33% and remaining 44% showed alteration in both MLH1 and MSH2 expression. (Table 16)

Table 16: Site of tumor in correlation with MMR protein loss

Site	Total	Lack of MLH1	Lack of MSH2	Lack of MLH1 and MSH2
Caecum	4	0	3(75%)	0
Asc. colon	4	0	1(25%)	1(25%)
Tr. colon	7	0	2(28%)	1(14%)
Desc. colon	3	0	1 (33%)	0
Sigmoid	10	0	2 (20%)	0
Rectum	**12**	**0**	**3 (25%)**	**2(16%)**

Loss of expression was more common on tumors on proximal colon compared to distal colon. (Graph 7)

Graph 7 : Site of tumor in correlation with MMR protein loss

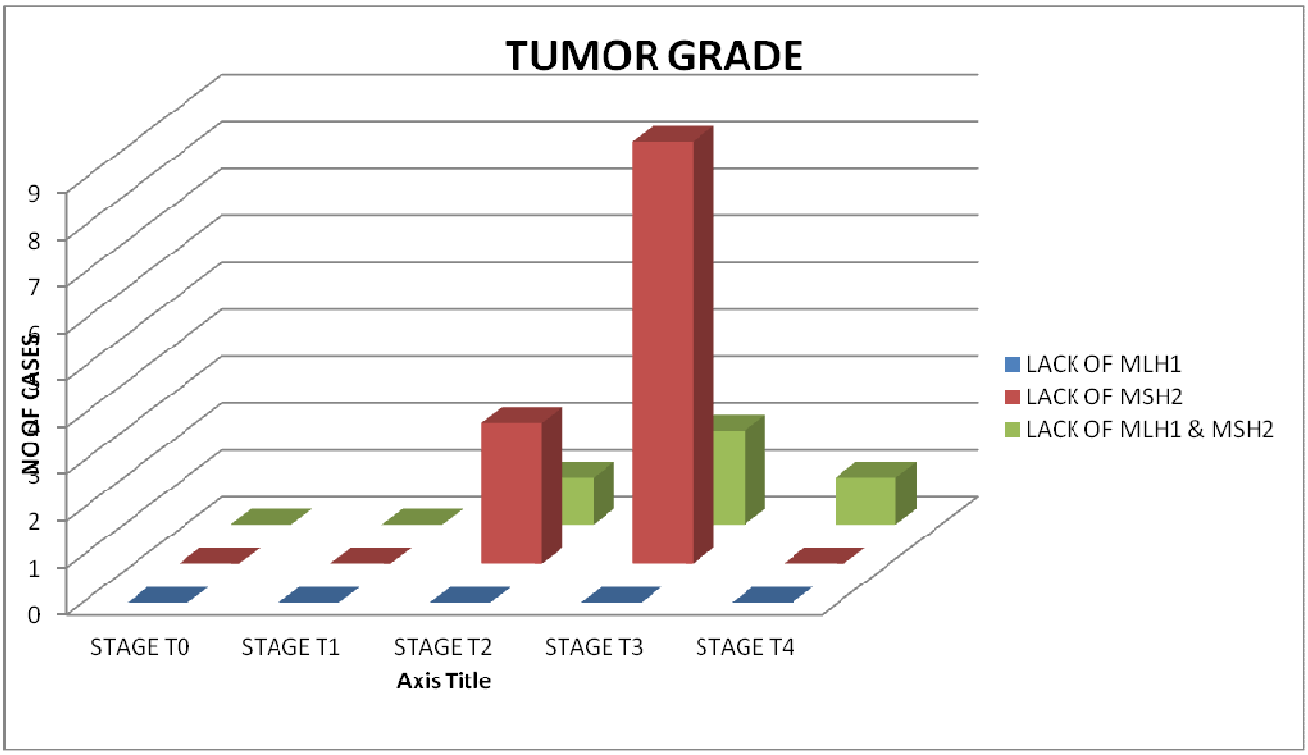


Of the 16 cases with alteration in MLH1 and MSH2, 11 cases were in T3 stage showing that the loss of expression was associated with higher stage. Among the 40 cases in study group, 28 were of T3 grade among which 11 showed lack of mismatch repair proteins. One case was in stage T4, which also showed lack of MLH1 and MSH2. (Table 17, graph 8)

Table 17: Stage of tumor in correlation with lack of MMR protein

Stage of the tumor	Total no	Lack of mlh1	Lack of msh2	Lack of mlh1 and msh2
To	0	0	0	0
T1	1	0	0	0
T2	10	0	3(30%)	1(10%)
T3	28	0	9(32%)	2(7%)
T4	1	0	0	1(100%)

Graph 8: Stage of tumor in correlation with MMR protein loss

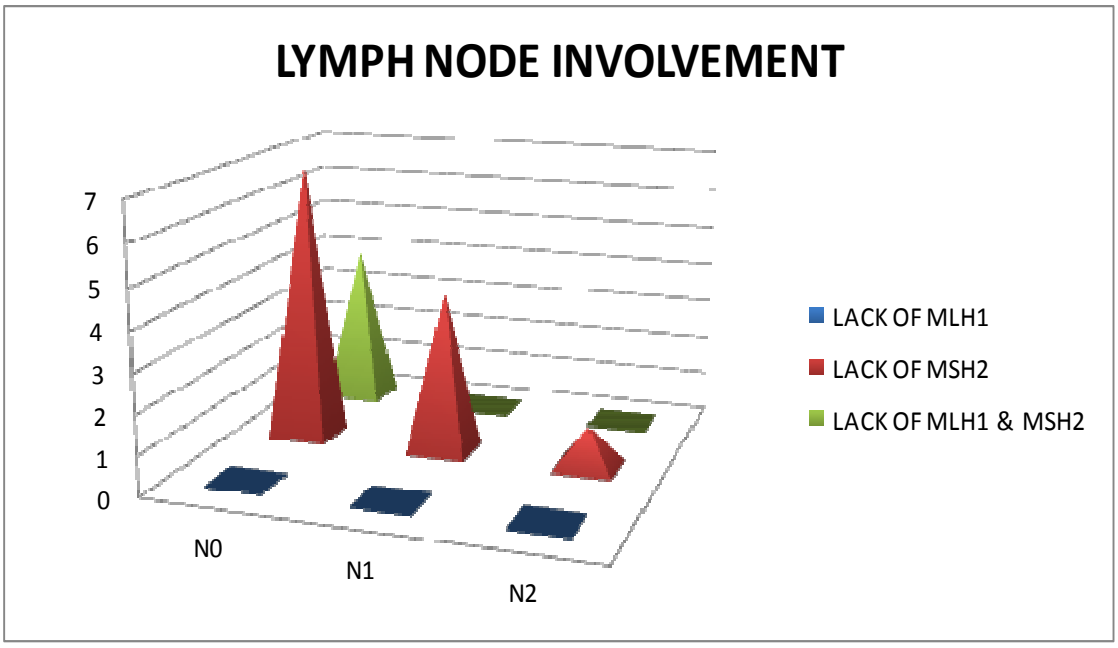


In our study expression of MLH1 and MSH2 did not have significant correlation with the lymph node involvement. Of the 40 cases, 29 cases were in N0 stage, 8 cases were in N1 and 3cases were in N2 stage. All the four cases with lack of both MLH1 and MSH2 were with no nodal metastases (N0). Table 18 and graph 9 depicts the correlation of lymph node involvement and expression of MMR proteins.

Table 18: Lymph node involvement by the tumor in correlation with MMR protein loss

Node involvement	Total no of cases	Lack of mlh1	Lack of msh2	Lack of mlh1 and msh2
N0	29	0	7(24%)	4(14%)
N1	8	0	4(50%)	0
N2	3	0	1(33%)	0

Graph 9: Lymph node involvement by the tumor in correlation with lack of MMR protein



STATISTICAL DATA

Method of sampling: Systematic Random Sampling

Research design: Descriptive study

The data obtained were entered in the master chart , coded and edited. The inferential and descriptive analysis of these data were computed using a software SSPS-17. The association between the variables and the lack of expression of MLH1 and MSH2 were determined by using chi- square test.

Association between sex and loss of MLH1 and MSH2:

Table 19 - Sex & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Sex	Male	Count	7	1	13	21
		% within Sex	33.3%	4.8%	61.9%	100.0%
		% within Lack of Expression	58.3%	25.0%	54.2%	52.5%
	Female	Count	5	3	11	19
		% within Sex	26.3%	15.8%	57.9%	100.0%
		% within Lack of Expression	41.7%	75.0%	45.8%	47.5%
Total		Count	12	4	24	40
		% within Sex	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

In the previous table the chi- square value 1.40 for the association between gender and lack of expression of MLH1 and MSH2 is not significant (p =0.496) revealing that there is no association between age and the MMR protein loss. (Table 19)

Association between age and MMR protein loss:

Table 20: Age in Years & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Years	Less than 40 years	Count	0	1	3	4
		% within Years	.0%	25.0%	75.0%	100.0%
		% within Lack of Expression	.0%	25.0%	12.5%	10.0%
	41 - 50 years	Count	4	0	5	9
		% within Years	44.4%	.0%	55.6%	100.0%
		% within Lack of Expression	33.3%	.0%	20.8%	22.5%
	51 - 60 years	Count	2	0	6	8
		% within Years	25.0%	.0%	75.0%	100.0%
		% within Lack of Expression	16.7%	.0%	25.0%	20.0%
	61 - 70 years	Count	0	1	7	8
		% within Years	.0%	12.5%	87.5%	100.0%
		% within Lack of Expression	.0%	25.0%	29.2%	20.0%
	71 - 80 years	Count	5	2	2	9
		% within Years	55.6%	22.2%	22.2%	100.0%
		% within Lack of Expression	41.7%	50.0%	8.3%	22.5%
	Above 81 years	Count	1	0	1	2
		% within Years	50.0%	.0%	50.0%	100.0%
		% within Lack of Expression	8.3%	.0%	4.2%	5.0%
Total		Count	12	4	24	40
		% within Years	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

In the previous table the chi- square value 14.375 for the association between age and lack of expression of MLH1 and MSH2 is not significant (p =0.157) revealing that there is no association between age and the MMR protein loss. (Table 20)

Association between size of the tumour and MMR protein loss:

Table 21 : Size & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Size	Less than 3 cm	Count	3	0	2	5
		% within Size	60.0%	.0%	40.0%	100.0%
		% within Lack of Expression	25.0%	.0%	8.3%	12.5%
	Between 3 - 6 cm	Count	8	2	19	29
		% within Size	27.6%	6.9%	65.5%	100.0%
		% within Lack of Expression	66.7%	50.0%	79.2%	72.5%
	Above 3 cm	Count	1	2	3	6
		% within Size	16.7%	33.3%	50.0%	100.0%
		% within Lack of Expression	8.3%	50.0%	12.5%	15.0%
Total		Count	12	4	24	40
		% within Size	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

From the above table there found to be no association between the tumor size and lack of MMR protein expression , the chi square value being 6.53 and p=0.162. (Table 21)

Association between histological type and lack of MMR expression:

The chi- square value for the association between these two variables is 10.625 and is significant($p<0.05$) revealing a strong association between the histological type of tumor and lack of expression of the MMR proteins.(Table 22)

Table 22: Histological type & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 an MSH2	Normal MLH and MSH2	Total
Histological type	Adenocarcinoma (conventional)	Count	6	4	22	32
		% within type	18.8%	12.5%	68.8%	100.0%
		% within Lack of Expression	50.0%	100.0%	91.7%	80.0%
	Mucinous Adenocarcinoma	Count	5	0	1	6
		% within type	83.3%	.0%	16.7%	100.0%
		% within Lack of Expression	41.7%	.0%	4.2%	15.0%
	Signet Ring cell Carcinoma	Count	1	0	1	2
		% within type	50.0%	.0%	50.0%	100.0%
		% within Lack of Expression	8.3%	.0%	4.2%	5.0%
Total		Count	12	4	24	40
		% within type	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

Association between degree of differentiation and MMR protein loss:

Table 23: Differentiation of Tumor & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Tumor	Well Differentiated	Count	2	0	2	4
		% within Tumor	50.0%	.0%	50.0%	100.0%
		% within Lack of Expression	16.7%	.0%	8.3%	10.0%
	Moderately Differentiated	Count	4	3	20	27
		% within Tumor	14.8%	11.1%	74.1%	100.0%
		% within Lack of Expression	33.3%	75.0%	83.3%	67.5%
	Poorly Differentiated	Count	6	1	2	9
		% within Tumor	66.7%	11.1%	22.2%	100.0%
		% within Lack of Expression	50.0%	25.0%	8.3%	22.5%
Total		Count	12	4	24	40
		% within Tumor	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

In the above table the chi- square value 10.185 for the association between degree of differentiation and lack of expression of MLH1 and MSH2 is not significant ($p < 0.05$) revealing that there is an association between degree of differentiation and the MMR protein loss.(Table 23)

Association between site and lack of MMR proteins:

Table 24 : Site & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Site	Caecum	Count	3	0	1	4
		% within Site	75.0%	.0%	25.0%	100.0%
		% within Lack of Expression	25.0%	.0%	4.2%	10.0%
	Asc. colon	Count	1	1	2	4
		% within Site	25.0%	25.0%	50.0%	100.0%
		% within Lack of Expression	8.3%	25.0%	8.3%	10.0%
	Tr. colon	Count	2	1	4	7
		% within Site	28.6%	14.3%	57.1%	100.0%
		% within Lack of Expression	16.7%	25.0%	16.7%	17.5%
	Desc. colon	Count	1	0	2	3
		% within Site	33.3%	.0%	66.7%	100.0%
		% within Lack of Expression	8.3%	.0%	8.3%	7.5%
	Sigmoid	Count	2	0	8	10
		% within Site	20.0%	.0%	80.0%	100.0%
		% within Lack of Expression	16.7%	.0%	33.3%	25.0%
	Rectum	Count	3	2	7	12
		% within Site	25.0%	16.7%	58.3%	100.0%
		% within Lack of Expression	25.0%	50.0%	29.2%	30.0%
Total		Count	12	4	24	40
		% within Site	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

In the above table the chi- square value 8.03for the association between site of tumor and lack of expression of MLH1 and MSH2 is not significant (p =0.626)

revealing that there is no association between site and the MMR protein loss. (Table 24)

Association between tumor stage and lack of MMR proteins:

Table 25: Stage of Tumor&Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Stage of Tumor	T1	Count	0	0	1	1
		% within Stage of Tumor	.0%	.0%	100.0%	100.0%
		% within Lack of Expression	.0%	.0%	4.2%	2.5%
	T2	Count	3	1	6	10
		% within Stage of Tumor	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	25.0%	25.0%	25.0%	25.0%
	T3	Count	9	2	17	28
		% within Stage of Tumor	32.1%	7.1%	60.7%	100.0%
		% within Lack of Expression	75.0%	50.0%	70.8%	70.0%
	T4	Count	0	1	0	1
		% within Stage of Tumor	.0%	100.0%	.0%	100.0%
		% within Lack of Expression	.0%	25.0%	.0%	2.5%
Total		Count	12	4	24	40
		% within Stage of Tumor	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

From the above table there found to be no association between the tumor stage and lack of MMR protein expression , the chi square value being 9.94 and p=0.127. (Table 25)

Association between the nodal involvement and MMR protein loss:

Table 26: Node involvement & Lack of Expression Crosstabulation

			Lack of Expression			
			MSH2	MLH1 and MSH2	Normal MLH1 and MSH2	Total
Node involvement	N0	Count	7	4	18	29
		% within Node involvement	24.1%	13.8%	62.1%	100.0%
		% within Lack of Expression	58.3%	100.0%	75.0%	72.5%
	N1	Count	4	0	4	8
		% within Node involvement	50.0%	.0%	50.0%	100.0%
		% within Lack of Expression	33.3%	.0%	16.7%	20.0%
	N2	Count	1	0	2	3
		% within Node involvement	33.3%	.0%	66.7%	100.0%
		% within Lack of Expression	8.3%	.0%	8.3%	7.5%
Total		Count	12	4	24	40
		% within Node involvement	30.0%	10.0%	60.0%	100.0%
		% within Lack of Expression	100.0%	100.0%	100.0%	100.0%

In the above table the chi- square value 3.10 for the association between lymph node involvement and lack of expression of MLH1 and MSH2 is not significant (p =0.541) revealing that there is no association between lymph node metastases and the MMR protein loss. (Table 26).

DISCUSSION

Colorectal cancer is the third most common type of malignancy in western countries. 15% of sporadic cases are due to MSI. In MSI, there are frame shift mutations and base pair substitutions in microsatellites. Microsatellites are repetitive genetic loci with 1 to 5 base pairs repeated 15 to 30 times. These mutations in microsatellites occur mainly during DNA replication and are normally controlled by the DNA mismatch repair genes such as MLH1, MSH2, MSH6, PMS2 and MSH3. MLH1 recruits its binding partner PMS2 to the site of DNA injury, so if the expression of MLH1 is lost then PMS2 will also be lost. Similarly, when MSH2 is lost, MSH6 will be lost. Several studies have reported that defect in MMR genes forms the basis for MSI. MSI is seen in all cases of HNPCC and a subset of sporadic colorectal carcinoma. [55] Mutations of MLH1 and MSH2 are more common and they occur in exon 16 and exon 12 of the genes respectively. It is important to screen all colorectal carcinomas for MSI, regardless of patient's age or family history. This helps in detecting sporadic cases with MMR protein defect and also potential cases of Lynch syndrome.

Sporadic tumors with microsatellite instability have a better prognosis when compared to microsatellite stable tumors (MSS). MSI tumors may respond less

favorably to 5- fluorouracil- based chemotherapy. Therefore, the knowledge of MSI status may pave the way to assess the prognosis and for therapy.

The screening can be done either by molecular methods or by immunohistochemical marker study for MSI and MMR protein. Molecular method is considered to be the gold standard for diagnosing mismatch repair genes but several recent studies have shown > 95% specificity of immunohistochemical analysis for mismatch repair genes.

The present study is an attempt to determine the frequency of loss of mismatch repair protein (MLH1 and MSH2) expression by immunohistochemical analysis and to study its correlation with various clinicopathologic characteristics. IHC determination of MLHI and MSH2 is becoming a more popular technique as it can define the MMR status in paraffin embedded tissue. Immunohistochemical analysis is less expensive and less time consuming when compared to molecular analysis. It is less labour intensive and is readily available to most diagnostic anatomic pathology laboratories while molecular testing is not. Other advantage with IHC is the availability of tissues to evaluate histopathological features of the tumors.[56]

We observed that in our study population there is 10% cases(4/40) lacked the expression of both the mismatch repair proteins. It was also observed that isolated loss of MSH2 was seen in 30% (12/40) cases. This is significantly higher when compared to the study done by Vijay pandey et al which quotes an incidence of 17% in an Indian cohort. Our literature search using google search engine showed only a few similar studies done in the Indian subcontinent. [39]

In the present study we also correlated various clinico-pathological features with MMR expression. Clinicopathological features studied include age of the patient, sex of the patient , location of the tumor, histological type, grade, stage of the tumor (T-stage) and lymph node involvement. The purpose of this was to establish any association between MMR expression and these variables.

In our study , the study population encompassed patients with age range between 33 and 85 years with a mean age of 58.9 years. 13 cases were below 50 years of age and the remaining 27 cases were above 50 years of age. The male to female ratio was 1.1:1 (21:19). Majority of the tumors were located in the distal colon, mostly from rectum which was in agreement with the previous study. [39] The sizes of the tumors varied from 1.0 cm to 8.0 cm in greatest dimension. Out of 40 cases, 32 were conventional adenocarcinomas, 6 were mucinous adenocarcinomas and 2

cases were signet ring cell type carcinomas. With respect to differentiation, 4 cases were well differentiated, 27 cases were moderately differentiated and 9 cases were poorly differentiated , including mucinous carcinomas and signet ring cell carcinomas. Lymph node metastases were noted in eleven cases.

While correlating the loss of expression of MLH1 and MSH2 with various clinicopathologic characters , we found that lack of expression of MSH2 and both of MLH1 and MSH2 were more often seen in females (46%) when compared to males(38%), similar to the study by K.Ohrling. They had stated that there is a relationship between the lack of MMR gene and gender.[56] However in our statistical analysis, we found that there is no significant (p=0.496) association between these two variables as evolved by a chi- square value of 1.40 .

In our study MMR defective tumors developed in the age group between 35 years and 85 years with a more frequent incidence in the 7^{th} decade. This was in correlation with the study of Rodrigo jover et al [55] where the mean age at diagnosis was high (70.5 yrs). Yet another study by Valerie Rigau showed that loss of MLH1 expression was frequent in elderly women. This proves that advanced age at the time of diagnosis does not rule out the possibility of hereditary non-polyposis colorectal carcinoma (HNPCC). [57] In our statistical analysis, the chi- square value 14.375 for the association between age and lack of expression of

MLH1 and MSH2 is not significant (p =0.157) revealing that there is no association between age and the MMR protein loss.

In the present study majority of the tumors (29/40) were between 3and 6 cm in size and 5 cases were less than 3cm in size. Of the 5 cases which are smaller than 3 cm in size, 3 cases (60%) showed isolated negative staining for MSH2 protein. Of the six cases larger than 6 cm in size only one case showed lack of MSH2 protein and two cases showed alteration in expression of both the proteins. This finding was different with previously reported studies which states that MSI tumors more often display larger size.[49] But in our study statistical analysis did not show any association between the tumor size and lack of MMR protein expression, the chi square value being 6.53 and p=0.162.

Tumors occurring in the caecum, ascending colon and transverse colon were classified as proximal colorectal carcinomas and tumors in the descending colon, sigmoid colon and rectum were classified as distal colorectal carcinomas. In our study, lack of MLH1 and MSH2 was more common on tumors on proximal colon compared to distal colon. In the present study, abnormality of MMR protein expression was observed in 53.3% of the proximal colorectal carcinomas when compared to only 32% of distal colorectal carcinomas. Few studies correlating

with these findings have been recorded in literature. The various studies suggested that early onset microsatellite instable tumors were located in proximal colon and mostly showed a poorly differentiated grade. Eventhough the lack of expression of MMR protein was more among the tumors on proximal colon, statistically it was not significant.[58,59] The chi- square value of 8.03for the association between site of tumor and lack of expression of MLH1 and MSH2 is not significant (p =0.626) revealing that there is no association between site and the MMR protein loss.

With regard to the histological type of the tumor, lack of MSH2 protein was more associated with mucinous adenocarcinoma (83%) and signet ring cell carcinoma (50%) compared to conventional adenocarcinoma. In this series, only19% of conventional adenocarcinoma showed loss of expression of MSH2 and 12.5% showed lack of expression both MLH1 and MSH2 protein. This was in agreement with a previous study done by Roberta Gafa et al [60] , where it was found that in a series of 216 cases, MSI tumors were found to be closely related to poorly differentiated tumors and mostly in mucinous adenocarcinoma.This would suggest a correlation between histologic type of the tumor and MMR protein expression. Statiscal analysis showed that there is a strong association between the histological type and MMR protein loss.The chi- square value for the association between these two variables was found to be 10.625 and is significant ($p<0.05$).

MMR protein expression was also found to be closely related to grade of the tumor. Of the 9cases of poorly differentiated adenocarcinomas, 6 cases showed loss of expression of MSH2 protein and one case showed lack of both MLH1 and MSH2 protein. The association between degree of differentiation and expression of MLH1 and MSH2 was found to be statistically significant. The chi-square value for these two variables is 10.625 and is significant ($p<0.05$) revealing a strong association. Most of the well differentiated tumors were positive for MMR protein, only 26% of cases showed deficient MMR protein.

A Previously reported study showed poorly differentiated tumors was associated with loss of MLH1 and the mucinous and medullary carcinomas were more frequently MLH1 negative and MSH2 positive.[57] Our present study however has contradicted these observations. In our study, poorly differentiated tumors including the mucinous adenocarcinomas were more often MSH2 negative.

Adrian Gologan did a study to review the performance of the revised Bethesda guidelines. They had also studied various histological features and found that all the MSI cases were in more advanced stages (T2 and T3).[43] In our present study there was only one case each in stage T1 and stage T4 and were no cases in stage T0. One case in stage T1 was positive for mismatch repair protein and the other case in stage T4 was mismatch repair protein negative. Tumors in stage 2 and 3 showed lack of expression of MMR protein 40% and 39% respectively.

Statistically also there was no association between the tumor stage and lack of MMR protein expression, the chi square value being 9.94 and p=0.127.

We were unable to establish a statistical correlation between MLH1 and MSH2 expression and lymph node involvement. The chi- square value 3.10 for the association between lymph node involvement and lack of expression of MLH1 and MSH2 was not significant (p =0.541). In our study 1/ 3 cases in N2 stage and 4/8 cases in N1 stage showed lack of MSH2 protein. One of the reasons could have been the less number of cases with lymph node metastasis. However no correlation between lymph node involvement and lack of MMR protein has been recorded in literature.

Screening for MSI helps in detecting sporadic cases with MMR protein defect and also potential cases of Lynch syndrome. In the present study 40% (16/40) cases showed lack of expression of either MSH2 or both MLH1 and MSH2 by immunohistochemical method. Among the various variables studied, only histological type and grade of the tumor showed statistically significant correlation with lack of mismatch repair protein. In colorectal carcinomas with MSI the common deficit proteins are MLH1 and MSH2. However a small proportion of MSI colorectal tumors are due to mutations in other MMR genes like MSH6, PMS1 and PMS 2. Hence, addition of antibodies against these proteins is also recommended in screening of colorectal carcinomas with MSI.

SUMMARY AND CONCLUSION

- 40% of the colorectal cancers studied lacked the expression of mismatch repair proteins (MMR) ,MLH1 and MSH2 which is significantly high when compared to other studies done in India.
- Loss of MMR protein was found to occur in older age group(7^{th} decade).
- Among the various variables studied, only histological type and grade of the tumor showed statistically significant correlation.
- MSH2 negative tumors were mostly poorly differentiated adenocarcinomas which included mucinous and signet ring cell carcinomas.
- Immunohistochemical staining for MMR proteins can be used as a reliable tool to detect MSI in colorectal cancers. This will help in the identification of these patients whose relatives need to be screened periodically to prevent the occurrence of colorectal cancer in them.
- The current study has tested only for the two common MMR proteins. Further studies are required to identify the occurrence of rare MMR proteins also.

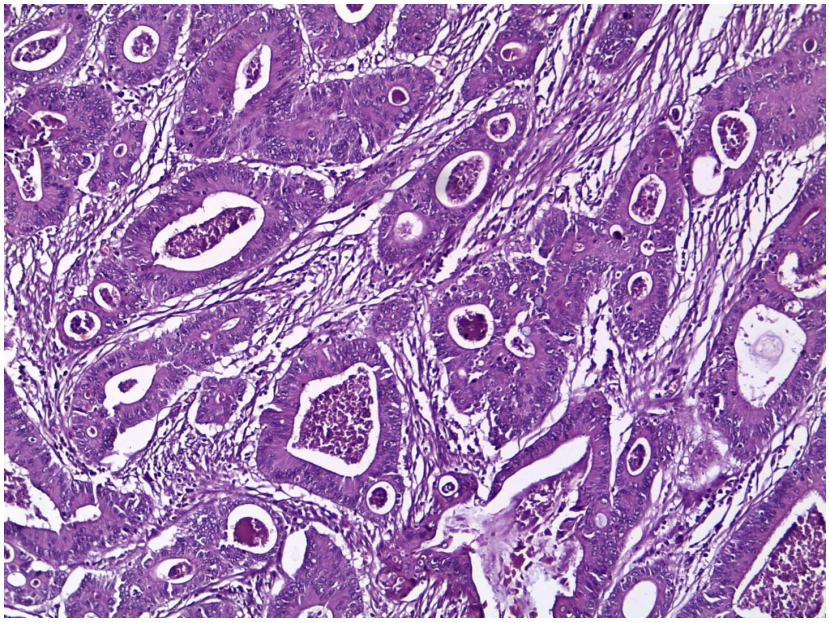


Fig 2: Well differentiated adenocarcinoma (H&E, 10X)

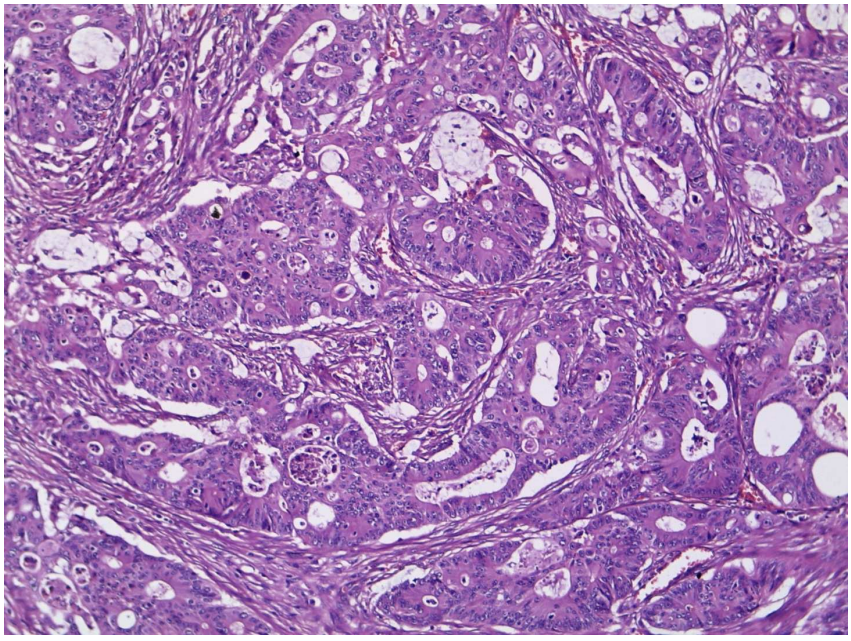


Fig 3: moderately differentiated adenocarcinoma (H & E, 10X)

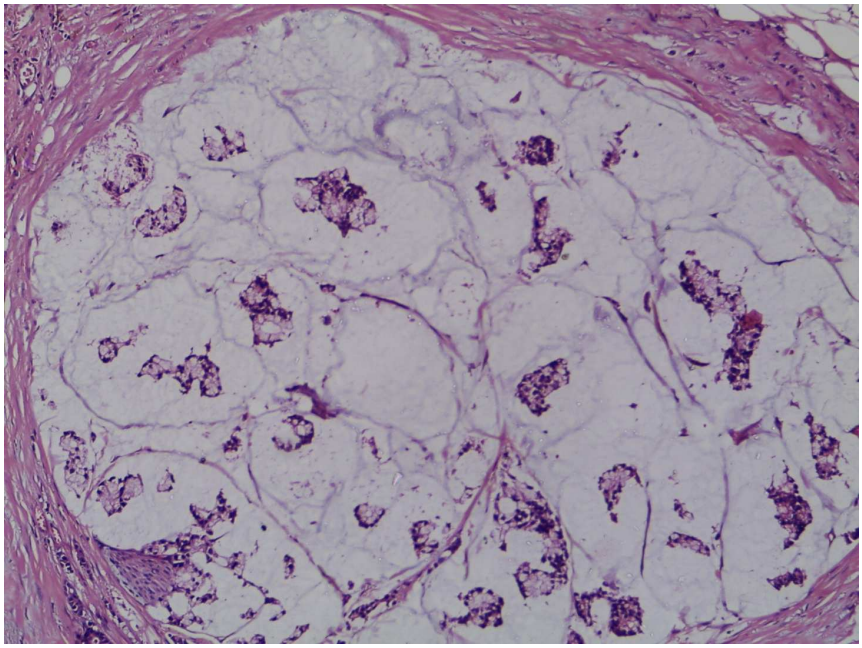


Fig 4: Mucinous adenocarcinoma (H &E , 10X)

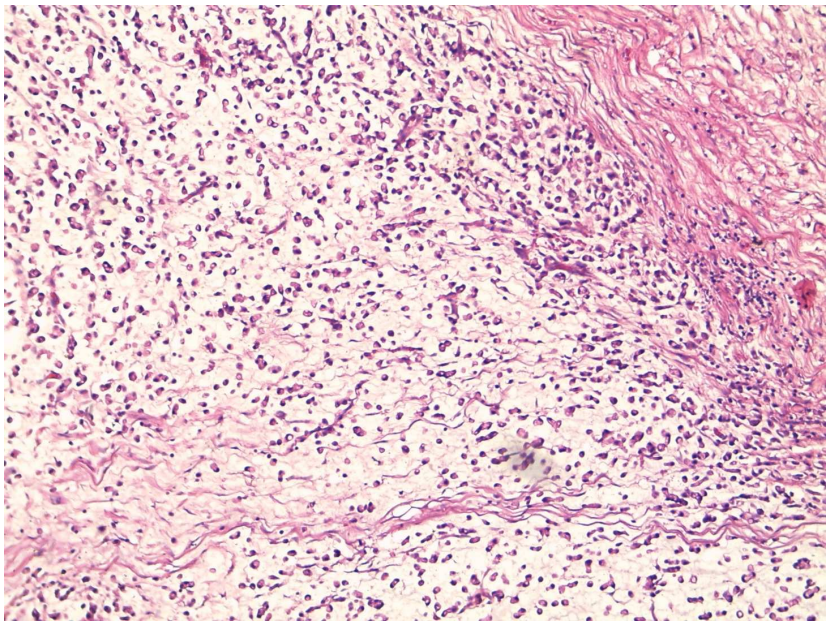


Fig 5: Signet ring cell carcinoma (H&E , 10X)

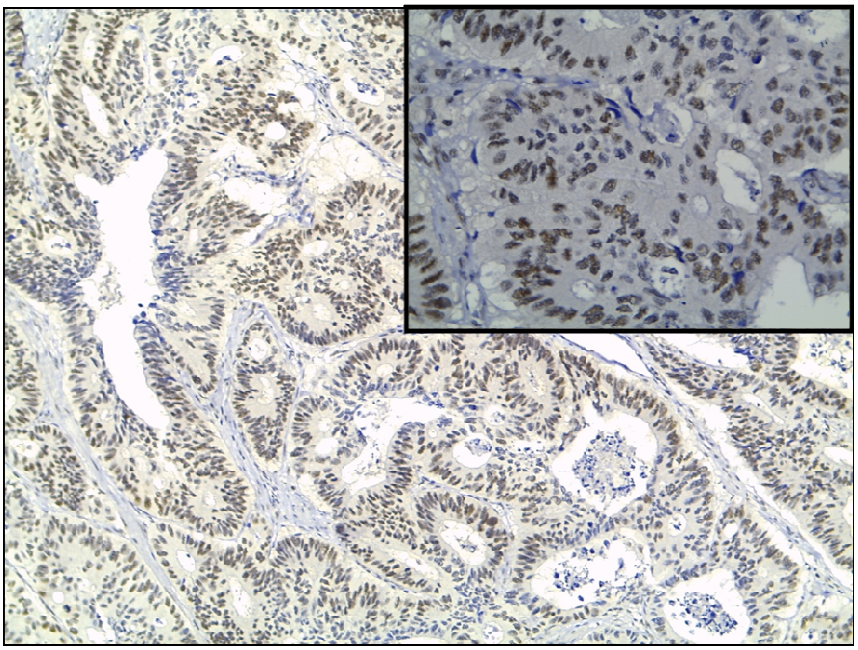


Fig 6: Moderately differentiated adenocarcinoma with MLH1 nuclear positivity (IHC,10x). Inset shows high power view (IHC, 40x)

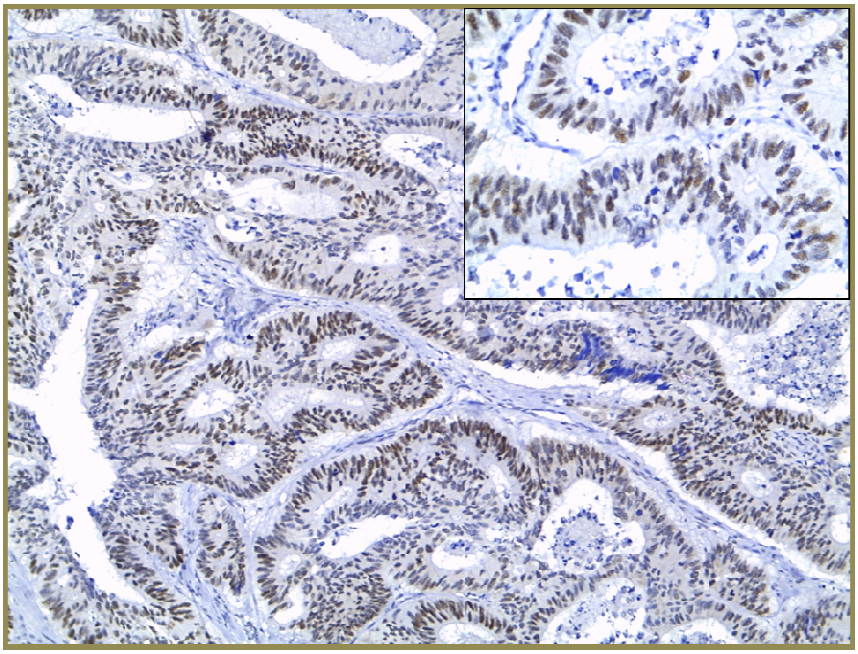


Fig 7: Moderately differentiated adenocarcinoma with MSH2 nuclear positivity (IHC,10x). Inset shows high power view (IHC, 40x)

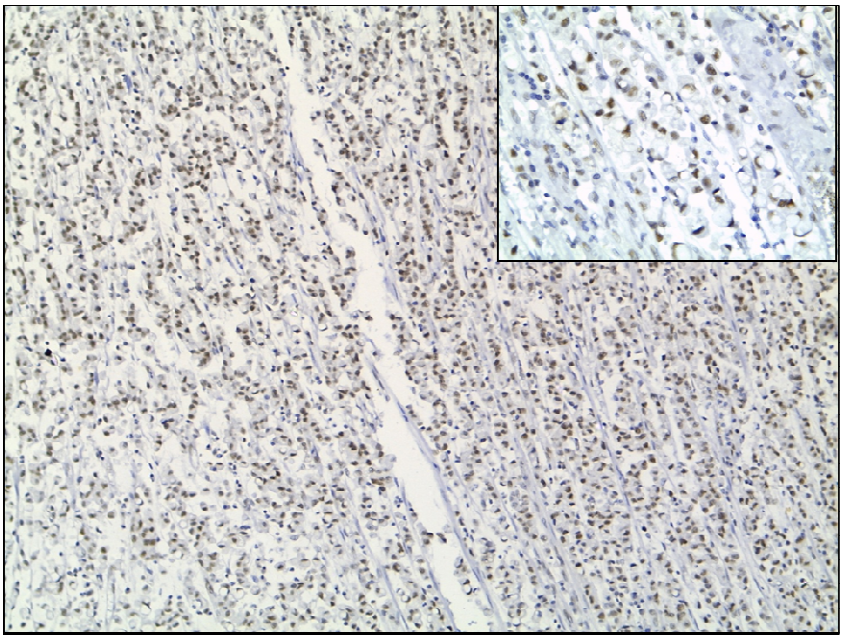


Fig 10: Signet ring cell carcinoma with MLH1 nuclear positivity(IHC,10X). Inset shows high power view.(IHC,40X).

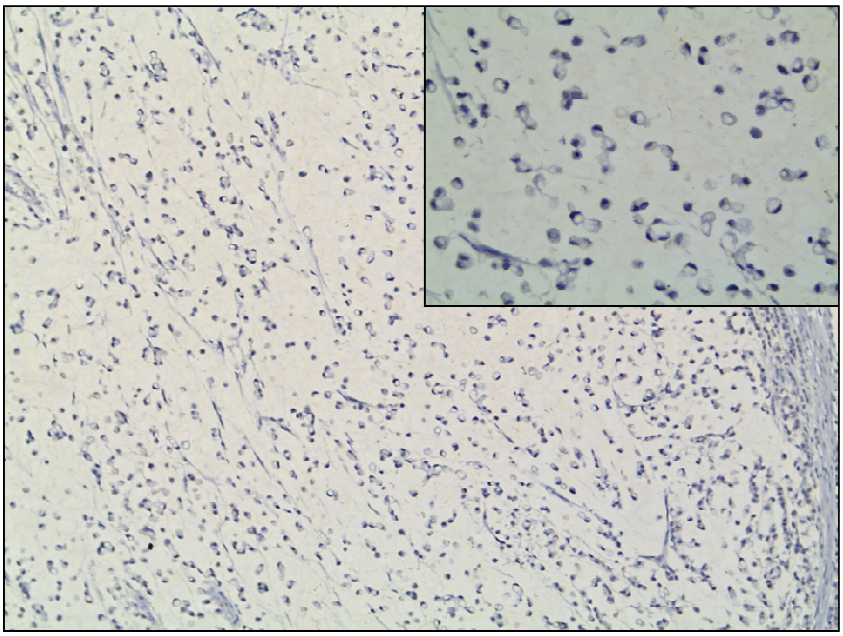


Fig 11: Signet ring cell carcinoma with negative MSH2 staining.(IHC,10X). Inset shows high power view(IHC,40X)

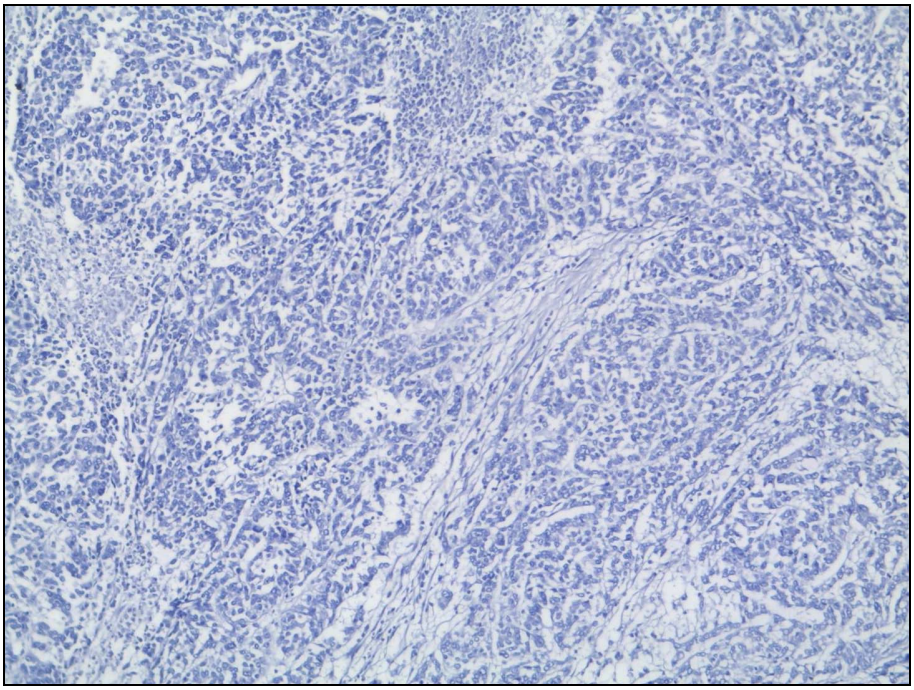


Fig 8: Poorly differentiated adenocarcinoma with negative MLH1 staining (IHC,10x).

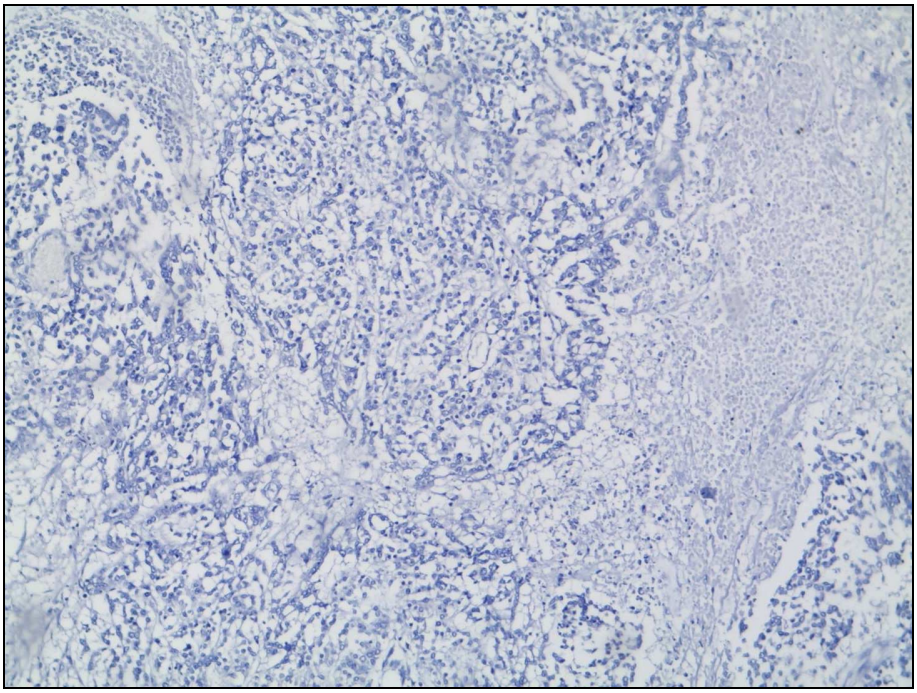


Fig 9: Poorly differentiated adenocarcinoma with negative MSH2 staining (IHC,10x).

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Master chart

s.no	HP No:	age	sex	Size of tumor	Site of tumor	grade	stage	Lymph node involvement	MLH1 expression	MSH2 expression
1	S337/10	83 years	male	4x3.5 cm	Sigmoid colon	Moderately differentiated	T2	N0	Positive	Positive
2	S883/10	55 years	female	6 cms along long axis	Rectum	Moderately differentiated	T3	N0	Positive	Positive
3	S979/10	74 years	male	5x3 cm	Transverse colon	Moderately differentiated	T3	N0	positive	Positive
4	S1792/10	69 years	Male	6x3 cm	Ascending colon	Well differentiated	T3	N1	positive	Positive
5	S1938/10	63 years	male	6x2 cm	Sigmoid colon	Mucinous adenocarcinoma	T3	N2	Positive	Positive
6	S1950/10	72 years	Male	5 cm along long axis	Ascending colon	Moderately differentiated	T3	N0	Positive	Positive
7	S2358/10	52 years	Female	1.3x1 cm	Rectum	Moderately differentiated	T2	N0	Positive	Positive

s. no	HP No	Age	Sex	Size of tumor	Site of tumor	Grade	Stage	Lymph node involvement	MLH1 expression	MSH2 expression
8	S2373/10	70 years	Male	3cm along long axis	Caecum	Signet ring cell caecinoma	T3	N2	Positive	Negative
9	S2658/10	53 years	Female	3x4 cm	Sigmoid colon	Moderately differentiated	T3	N1	Positive	Positive
10	S3076/10	78 years	Male	3.5 cm along long axis	Descending colon	Moderately differentiated	T3	N1	Positive	Negative
11	S3185/10	59 years	Male	8x4 cm	Sigmoid colon	Moderately differentiated	T3	N1	Positive	Positive
12	S3313/10	33 years	Female	3x2.8 cm	Rectum	Well differentiated	T2	N0	Positive	Positive
13	S3792/10	35 years	Female	9x3 cm	Transverse colon	Poorly differentiated adenocarcinoma	T4	N0	Negative	Negative

s.no	HP No	Age	Sex	Size of tumor	Site of tumor	Grade	Stage	Lymphnode involvement	MLH1 expression	MSH2
14	S3817/10	76 years	Male	5x4 cm	Ascending colon	Moderately differentiated	T3	N0	Negative	Negative
15	S4301/10	44 years	Male	4x4 cm	Transverse colon	Mucinous adenocarcinoma	T3	N1	Positive	Negative
16	S4542/10	71 years	Male	4 cm along long axis	Caecum	Moderately differentiated	T3	N0	Positive	Positive
17	S3912/10	43 years	Female	2 cm along long axis	Sigmoid colon	Moderately differentiated	T3	N0	Positive	Negative
18	S2793/11	52 years	Female	8 cm along long axis	Sigmoid colon	Moderately differentiated	T3	N0	Positive	Positive
19	S1932/11	35 years	Female	3 cm along long axis	Rectum	Moderately differentiated	T3	N0	Positive	Positive

s.no	HP No		sex	Size of tumor	Site of tumor	Grade	stage	Lymph node involvement	MLH1 expression	MSH2 expression
20	S45/11	78 years	Female	1 cm stricture	Caecum	Well differentiated	T2	N0	Positive	Negative
21	S646/11	55 years	Female	5cm along long axis	Transverse colon	Moderately differentiated	T2	N0	Positive	Positive
22	S2545/11	61 years	Male	2x2.5 cm	Sigmoid colon	Moderately differentiated	T1	N0	Positive	Positive
23	S2487/11	70 years	Male	6x4 cm	Transverse colon	Moderately differentiated	T2	N0	Positive	Positive
24	S332/09	70 years	Male	6.5x5 cm	Rectum	Moderately differentiated	T3	N0	Positive	Negative
25	S3209/09	49 years	Male	4x3 cm	Rectum	Well differentiated	T2	N0	Positive	Negative

s.no	HP No	age	sex	Size of tumor	Site of tumor	grade	stage	Lymph node involvement	MLH1 expression	MSH2 expression
26	S2655/09	52 years	Male	4.5x3.5 cm	Ascending colon	Mucinous adenocarcinoma	T2	N0	Positive	Negative
27	S1067/09	43 years	Male	4.2x4 cm	Sigmoid colon	Moderately differentiated	T2	N0	Positive	Positive
28	S5041/09	38 years	Female	3.2x3 cm	rectum	Moderately differentiated	T3	N0	Positive	Positive
29	S400/09	42 years	Male	2.5x1 cm	Rectum	Moderately differentiated	T3	N2	Positive	Positive
30	S1427/09	50 years	Male	5.5x5 cm	Rectum	Moderately differentiated	T2	N0	Positive	Positive
31	S2766/08	43 years	Female	6x5 cm	Rectum	Moderately differentiated	T3	N1	Positive	Negative

s.no	HP No	age	sex	Size of tumor	Site of tumor	grade	stage	Lymph node involvement	MLH1 expression	MSH2 expression
32	S190/08	61 years	Female	8x6.5 cm	Rectum	Moderately differentiated	T2	N0	Negative	Negative
33	S4355/08	72 years	Female	6x4.5 cm	Sigmoid	Mucinous adenocarcinoma	T3	N0	Positive	Negative
34	S4169/08	58 years	Male	6x6.5 cm	Caecum	Mucinous adenocarcinoma	T3	N0	Positive	Negative
35	S4367/08	48 years	Female	3x2 cm	Rectum	Moderately differentiated	T3	N0	Positive	Positive
36	S1237/08	66 years	Female	6x5 cm	Descending colon	Moderately differentiated	T3	N0	Positive	Positive
37	S1944/09	75 years	Female	4x2.5 cm	Rectum	Moderately differentiated	T3	N0	Negative	Negative

s.no	HP No	age	sex	Size of tumor	Site of tumor	grade	stage	Lymph node involvement	MLH1 expression	MSH2 expression
38	S2757/09	45 years	Male	6x 4.5 cm	Transverse colon	Mucinous adenocarcinoma	T3	N1	Positive	Positive
39	S3178/09	85 years	Female	4.5x6 cm	Transverse colon	Signet ring cell carcinoma	T3	N1	Positive	Negative
40	S2062/10	78 years	Female	3.5 cm along long axis	Sigmoid colon	Moderately differentiated	T3	N0	Positive	Positive